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The Design, Synthesis and Screening of Potential Pyridinium Oxime Prodrugs (U)

Annual Report

Ronald T. Borchardt and John E. Simmons

February 29, 1984

Supported by

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University of Kansas
Center for Biomedical Research
Lawrence, Kansas 66044

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
In an attempt to improve the delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE) to the Central Nervous System (CNS), structural analogs and prodrugs of N-methylpyridinium 2-carbaldoxime (2-PAM) have been synthesized. The Series I prodrugs are dihydropyridinium oximes (pro-2-PAM's) which possess electron withdrawing substituents in the

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3- or 5-position. As precursors to these Series I prodrugs several 5-substituted-2-PAM's (I, Br substituted) and a 3-substituted 2-PAM (I-substituted) have been synthesized and characterized. In vitro and in vivo (mice) screening of these compounds indicates some promise as AChE regenerators, prompting the preparation of gram quantities of the I-substituted oximes for further biological evaluation. Efforts toward the synthesis of other 3- or 5-substituted (CN, $CONH_2$ substituted) oximes were initiated. The Series II prodrugs are tetrahydropyridinium oximes which possess a labile ring substituent. These mashed prodrugs (double latentiation) are addition products of pro-2-PAM. Several tetrahydropyridinium oximes (CN, SCN and Br substituents) have been synthesized and characterized. Conversion from the tetrahydropyridinium oximes to 2-PAM was measured under physiological conditions (pH 6.5-8.5) and only with the CN adduct was there appreciable conversion to 2-PAM.

To aid in the in vitro biological evaluation of the potential regenerators of AChE, a new screening assay was developed employing immobilized AChE. AChE covalently attached to functionalized polyethylene beads showed good esterase activity and was stable when stored at -16° C for up to 4 months. The AChE activity of the immobilized enzyme was continuously monitored spectrophotometrically in a closed loop fashion using acetylthiocholine and dithiobis (nitrobenzoic acid). The assay allowed for independent inactivation and reactivation of AChE, followed by the determination of regenerated AChE activity.

To aid in the in vivo biological evaluation of potential regenerators of AChE, a simple and reliable high performance liquid chromatography (HPLC) assay for pyridinium oximes in biological tissues was developed. The assay was sufficiently sensitive to allow for detection of 2-PAM in brain and other biological tissues.

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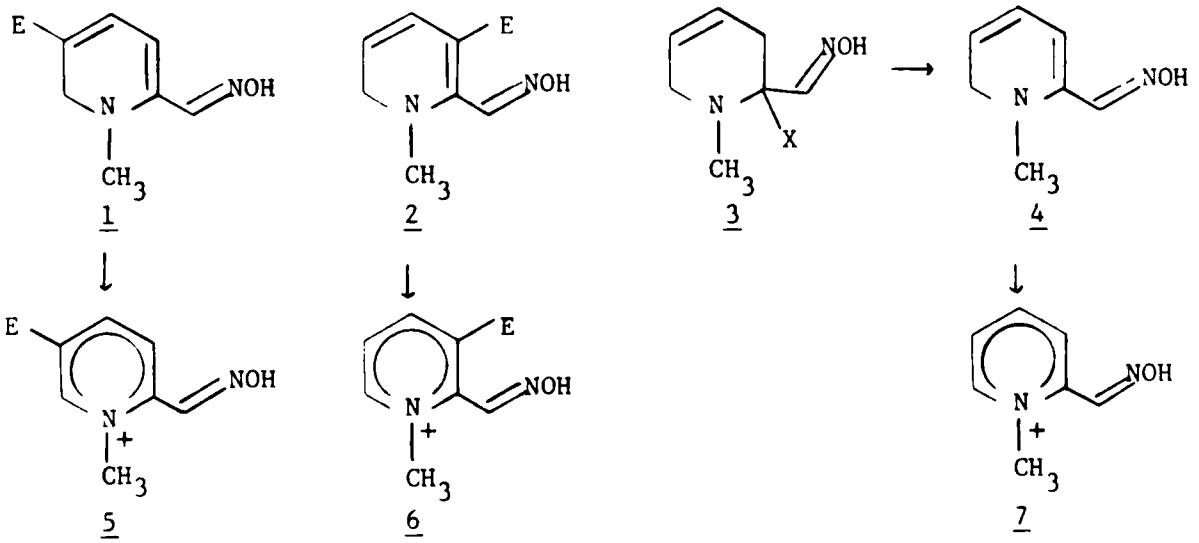
Summary

In an attempt to improve the CNS delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE), we have initiated chemical and biochemical studies on structural analogs and prodrug forms of N-methylpyridinium 2-carbaldoxime (2-PAM, 7). Over the past year we have concentrated our efforts on synthesizing two basic types of pro-PAM derivatives. Series I are dihydropyridinium oximes 1 and 2, which possess electron withdrawing substituents in the 3- or 5-position. These compounds require oxidation (latentiation) to generate the substituted 2-PAM's 5 and 6. It is hoped that the electron withdrawing substituent will stabilize the dihydropyridine structure, resulting in a slower in vivo conversion and thereby improving biodistribution. Series II are tetrahydropyridinium oximes 3, which possess a labile ring substituent. These masked prodrugs (double latentiation) are addition products of dihydropyridinium oxime 4 (pro-2-PAM) and require a two-step (elimination and oxidation) conversion to the active quaternary oxime 7 (2-PAM).



In series I the 5-substituted-2-PAM's 5 (E=I,Br) and a 3-substituted-2-PAM 6 (E=I) have been synthesized and characterized. In vitro (immobilized AChE) and in vivo (mice) screening of these compounds showed promising results and, therefore, gram quantities of the iodo-substituted oximes have been prepared for further biological evaluation. Efforts toward the synthesis of series I compounds 5 and 6 (E=CN,$CONH_2$) continue. In series II the tetrahydropyridinium oximes 3 (X=CN,SCN,Br) have been made and characterized. Conversions from 3 to 7 were measured in phosphate buffer (pH 6.5-8.5) and only when X=CN was there appreciable conversion to 2-PAM.

A screening assay employing immobilized AChE has been developed. AChE covalently attached to functionalized polyethylene beads showed good esterase activity and was stable when stored at -16°C for up to 4 months. The AChE activity of the immobilized enzyme could be continuously monitored spectrophotometrically in a closed loop fashion using acetylthiocholine and dithiobis(nitrobenzoic acid) (DTNB). The assay allows for independent inactivation and reactivation of AChE, followed by the determination of regenerated AChE activity. Both charged (2-PAM, TMB-4) and uncharged (monoisonitrosoacetone [MINA]) regenerators were used to evaluate and standardize the assay. 3/5-I-2-PAM's and 5-Br-2-PAM exhibited 50-60% of the activity of 2-PAM at 10^{-3} M in this screening assay. An ED_{50} determination (diisopropylfluorophosphate [DFP] challenge/mice) showed 5-I-2-PAM (5) to be significantly more effective than 2-PAM (7).

A simple and reliable HPLC assay for pyridinium oximes in biological tissues has been developed. An organic polymer based column packing (Brownlee PRP-1) with a wide pH tolerance allowed for elution with acidic (pH 2.5) and basic (pH 10.5) mobile phases. 2-PAM could be detected in the 40 picomole range (20 µl on column) with no interference from biologicals. The sensitivity allowed for the study of pyridinium oxime biodistribution, including brain levels, without the use of radiotracer techniques.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No.

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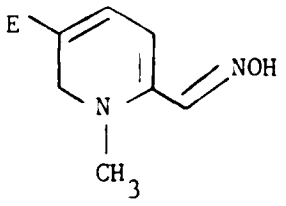
Tables and Figures

A. Problem

1. Design and Synthesis of Pro-PAM Agents

The overall objective of the project is to improve central nervous system (CNS) delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE). The specific chemical problem is to design and synthesize nonquaternary, lipophilic prodrugs of pyridinium oximes which can be easily transformed *in vivo* into active quaternary regenerators. Two basic prodrug designs are currently being investigated: a) Dihydropyridine carbaldoximes 1 and 2 substituted at the 3- or 5-position with an electron withdrawing group (E)-activation of these compounds to quaternary pyridinium oximes requires simple oxidation; b) Tetrahydropyridine oximes 3 substituted at the 2-position with a labile leaving group (X)-activation of these analogs requires elimination (-HX) followed by oxidation to generate quaternary pyridinium oximes.



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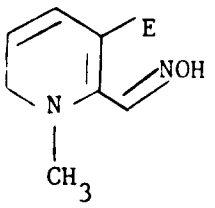

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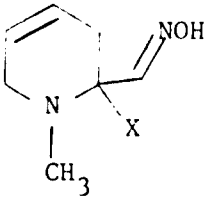

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Determination of the rate and efficiency of conversion from prodrug to parent pyridinium oxime forms will be required for evaluation of therapeutic potential and reactivator structural refinement. The measurement of physical properties such as pKa, partition coefficient and water solubility will be required to predict membrane penetration, tissue disposition and ability to dephosphorylate AChE.

2. Biological Testing

All drug candidates will require evaluation as regenerators of organophosphate-inhibited cholinesterase. Effective *in vitro* and *in vivo* screens will be necessary to evaluate both the parent pyridinium oximes and prodrug forms. In addition, a simple and effective pyridinium oxime detection technique for tissue distribution and elimination studies will be required. These studies will be essential in evaluating regenerator efficacy and CNS penetration.

B. Background

1. Regenerators

Organophosphates as a class owe their toxicity to their ability to react covalently with the esteratic site of AChE. AChE is the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine. The phosphorylated enzyme can be reactivated by a variety of agents. There is, however, a competing "aging" process whereby the inactivating phosphoryl group either migrates to an adjacent amino acid residue[1] or is partially hydrolyzed.[2,3] Phosphorylated AChE which has undergone this "aging" process is not easily reactivated. Thus rapid reactivation of the poisoned enzyme in all affected tissues is highly desirable.

I. B. Wilson discovered 2-PAM (7), which is now one of the most widely used and therapeutically effective broad spectrum AChE regenerators.[4] Wilson postulated that the electrostatic attraction of the quaternary nitrogen helped orient the oxime moiety toward the phosphorylated esteratic site (Figure 1).[4-6]

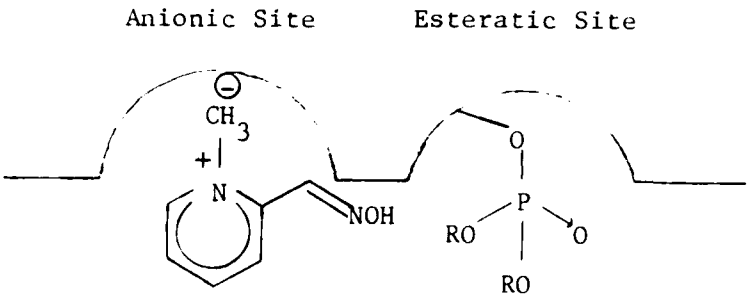
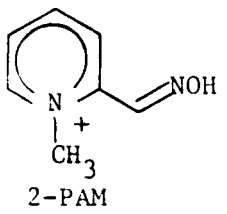


Figure 1. Proposed Active Site of AChE.

Since the discovery of 2-PAM, there have been a variety of other organic oximes which have been synthesized and screened for AChE regenerating activity. The charged bis oximes TMB-4 (8) and HI-6 (9) have both proven to be potent reactivators, but suffer from poor tissue penetration, short serum half lives and toxicity problems.[7] Neutral oximes such as MINA (10) and 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole (11) have shown much less potent regenerator ability, but do possess better lipid solubility.[8-10]



2-PAM

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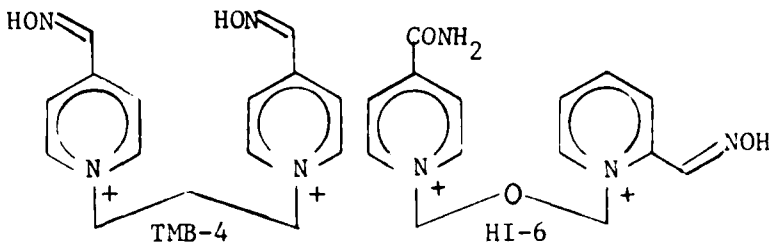

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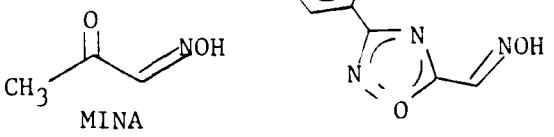

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Investigations into the structure activity relationships of substituted 2-PAM's has produced some interesting information. In general, electron withdrawing substituents shift the pKa of the pyridinium oxime to values below the optimum range of 7.4-7.8, and electron donating substituents shift it higher.[4,11] Some 5-substituted 2-PAM's (Cl, CH_3) were approximately as effective as 2-PAM in whole animal survival studies, even though in vitro testing showed them to be less effective at regenerating deactivated AChE.[12]

These results indicate that the active site of AChE can tolerate minor structural changes in 2-PAM and, further, that desirable distribution characteristics might be incorporated into the molecule without significantly reducing its reactivating capabilities. In an attempt to increase the lipid solubility of 2-PAM, N-dodecyl-2-pyridinium carbaldoxime (2-PAD) was made. It possessed increased lipid solubility, but proved to be far less effective than 2-PAM at in vitro reactivation.[13]

Only trace amounts of 2-PAM can be detected in the CNS following i.v. injection.[1] Due to its high water solubility, the intact drug is rapidly eliminated from the body with an observed half life in humans of less than one hour. Blood levels fall below the therapeutically effective range after 1-2 hours.[14,15] Significant brain levels of 2-PAM can only be achieved by intraventricular injections of the drug.[16,17]

Asphyxiation due to suppression of the central respiratory center is the ultimate cause of death in mammals exposed to anticholinesterase agents.[18,19] The lipid permeability of many organophosphates allows them to penetrate many body tissues, including the CNS, which are impermeable to a charged molecule like 2-PAM. Therefore, the need to regenerate AChE in the CNS presents an interesting drug delivery problem.

The delivery of 2-PAM to the highly lipid CNS was achieved with the pioneering work of Shek, Bodor and Higuchi.[20-23] The University of Kansas group synthesized a prodrug of 2-PAM. Working on the hypothesis that a tertiary amine would have little difficulty penetrating the CNS, they synthesized a partially reduced form of 2-PAM which was a latent quaternary amine. Figure 2 illustrates how they trapped the reduced form of 2-PAM as a cyanide addition product, 3a, which upon careful decomposition afforded pro-2-PAM (4). The pKa of 4 was determined to be 6.3, which was good for favorable physiological partitioning. They found that *in vivo* oxidation of 4 to 2-PAM (7), much like the NAD-NADH redox system, took place in approximately 1 minute.[22] This was sufficient time to allow the drug to cross the blood-brain barrier, producing a 13-fold increase in brain levels of 2-PAM.[23]

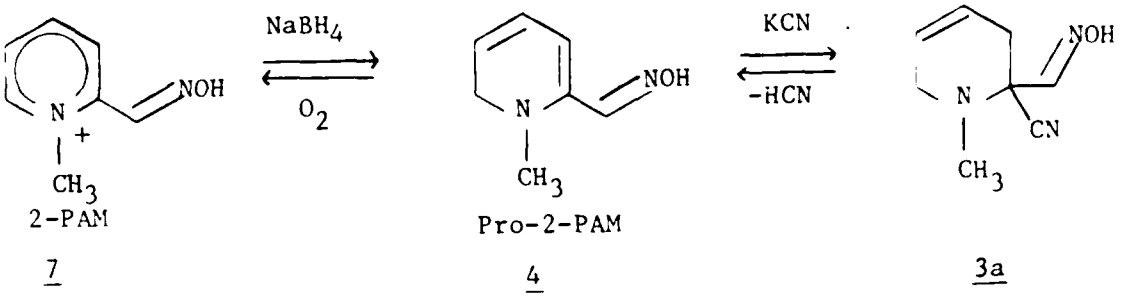


Figure 2. Synthesis of Pro-2-PAM.[20,23]

2. Biological Screening of Potential Regenerators

a. In Vitro Assays

Available *in vitro* screening techniques involve purified or partially purified soluble cholinesterase which is exposed to an organophosphate, and incubated with a regenerator, followed by the determination of enzyme hydrolytic activity. These assays generally involve the use of either acetylcholine or an analog which can be hydrolyzed by the enzyme. The resulting hydrolysis products can be measured colorimetrically, spectrally, and via pH change either directly or by CO_2 equilibria (both volumetric and radiometric).[8,9,24-27] By far the most convenient and sensitive method is that developed by Ellman et al.[28] which utilizes acetylthiocholine as substrate and measures the formation of thiocholine by its reaction with a disulfide chromogen.[28]

b. In Vivo Assays

In vivo evaluation of oxime regenerators has employed mice, rats, guinea pigs and rabbits.[29-31] The determination of LD_{50} values for potential regenerators as well as ED_{50} values ($2xLD_{50}$ challenge of organophosphate) are well documented and provide reliable, effective and comparable data. The determination of protective ratios (PR), in which doses of regenerators raise the lethal dose of organophosphates required to produce death, provides useful information about potential therapeutics.

3. Biodistribution

Biodistribution patterns of pyridinium oximes have been determined, using three basic techniques - the administration of radiolabeled oximes followed by paper chromatographic identification is, to date, the most sensitive technique.[23,32] UV spectrophotometric quantitation of oximes in tissue extracts has also been employed; however, this does not provide positive identification of the measured species.[33] More recent developments involve the use of high performance liquid chromatography to identify and quantitate quaternary oximes in body tissues.[34]

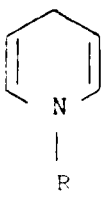
C. Approach

1. Prodrug Design

Our current work at the University of Kansas is intended to build on and exploit the initial findings of Shek, Bodor and Higuchi.[20-23] We have over the past year focused our efforts on two approaches to improved regenerators.

a. Series I - Dihydropyridinium Oximes

In the first series of compounds the intent was to reduce measurably the rate of oxidation of a dihydropyridinium oxime to its active parent quaternary form by stabilizing the dihydro-structure with electron withdrawing substituents. It is known from the literature that an electron withdrawing group in the 3- or 5- position stabilizes the dihydropyridines[35] 13 and 14, relative to 12, through electron delocalization.



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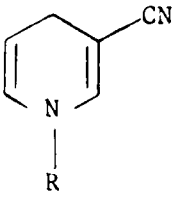

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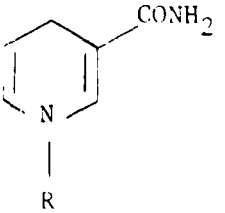

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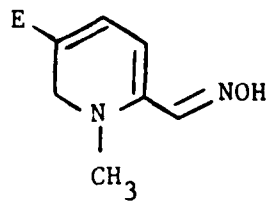
By synthesizing 3- and 5-substituted pro-2-PAM's 1-2, it is hoped that the



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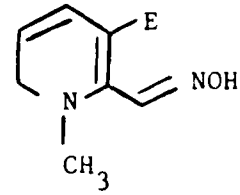

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E = I(a), Br(b), Cl(c), CN(d), $CONH_2$(e)

rate of conversion from prodrug to the active quaternary pyridinium oxime forms can be slowed, thus allowing more time for partitioning into the CNS compartment (Figure 3). There may very well be a tradeoff between reduced reactivating ability of the substituted pyri inium oxime and increased tissue permeability of the prodrug form; however, this can be effectively determined only by in vivo testing.

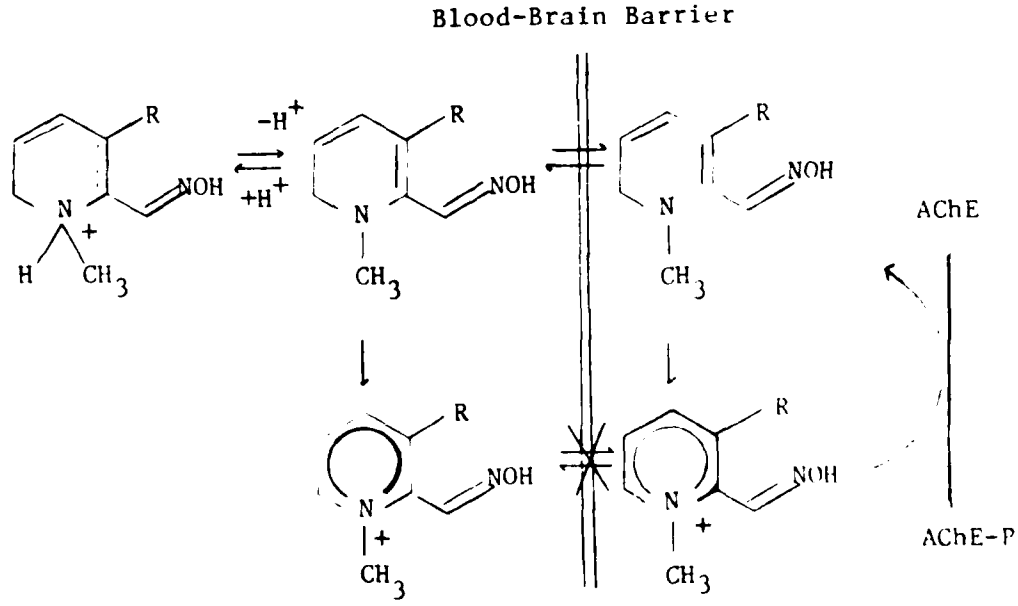

Figure 3. Pro-2-PAM Permeability of the Blood Brain Barrier and Conversion to 2-PAM.

b. Series II - Tetrahydropyridinium Oximes

The second series of compounds we have examined this past year are prodrugs which require a two step conversion to the active quaternary pyridinium oxime - double latentiation. Shek et al.[21] in their synthesis of Pro-2-PAM trapped the dihydropyridinium structure as a cyanide addition product 3a (Figure 2). Our intent is to take advantage of this trapping mechanism using nucleophiles (X) such as SCN, SO_3H, I, Br and OCN, which we hope will be as labile as, but less toxic than cyanide. These doubly latent structures 3 can generate pro-2-PAM (4) in vitro.

X = CN(a), SCN(b), Br(c), I(d), SO_3H(e), OCN(f)

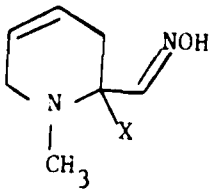

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Figure 4 illustrates how double latentiation provides two essentially neutral molecules (tetrahydro- and dihydropyridines), hence, two chances to cross membranes otherwise impenetrable to charged pyridinium oximes. Our goal has been to synthesize doubly latent forms of the parent oxime 2-PAM, since its regenerating capabilities and pharmacokinetics have been extensively documented in the literature.

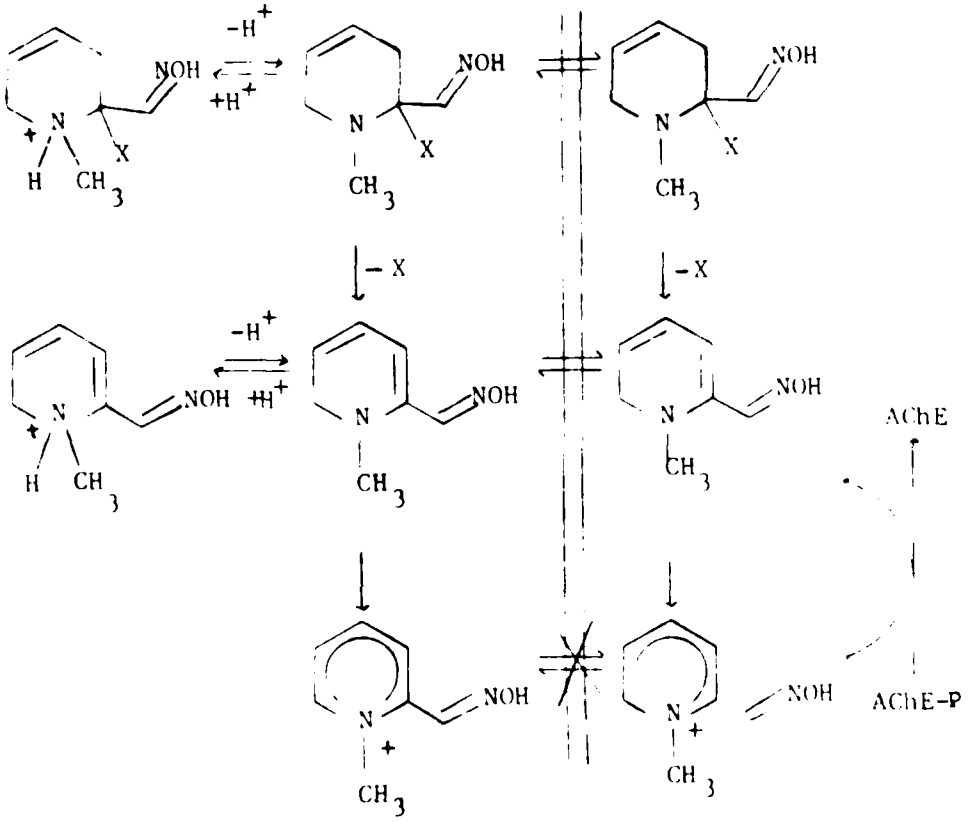


Figure 4. Tetrahydropyridinium Oxime Permeability of the Blood Brain Barrier and Conversion to 2-PAM.

2. Biological Evaluations

a. In Vitro

We have focused our attention on continued development and validation of a reliable and efficient in vitro AChE assay as a preliminary screening technique for the potential new regenerators being synthesized in our laboratories. The basic Ellman technique appeared to be a viable assay which did not require any dedicated equipment other than a spectrophotometer.[28] The intent was to have a rapid and efficient assay to be used as a primary screen for the parent quaternary pyridinium oximes. In this manner the quaternary oximes which exhibft poor or negligible reactivation capabilities compared to 2-PAM could be identified and discarded. This would allow concentration of efforts toward synthesizing prodrug forms of the most active regenerators. In addition, many of the prodrugs are oxygen sensitive; which necessitates that the assay be adaptable to anaerobic conditions. All of the above requirements (i.e. speed, reliability, sensitivity and adaptability to anaerobic conditions) led us to the Ellman technique to measure AChE activity and the use of an immobilized AChE. We also intend to extend the in vitro screening assay to incorporate whole brain tissue and thus establish the relationship between purified enzyme behavior and that of enzyme in a biomatrix.

b. In Vivo

Working with mice, we have focused on the determination of LD_{50} and ED_{50} values (VS $2xLD_{50}$ DFP challenge) of new regenerators. In addition, work on protective ratios should provide information helpful in evaluating new regenerators.

c. Biodistribution

Our efforts have focused on the development of assays, which allow for quantitative determination of tissue uptake and elimination patterns of pyridinium oximes. The use of high performance liquid chromatography and UV detection allows positive identification and quantitation of quaternary oximes and their metabolites in tissues. The examination of silicon based reversed-phase column eluted with aqueous/organic solvents containing ion pairing reagents for mobility control as well as hydrocarbon resin-based reversed-phase columns eluted with aqueous solutions at various pH's for mobility control allows identification of several general, universally applicable conditions for identification and quantitation of pyridinium oximes in all tissues, especially the CNS.

D. Results

1. Syntheses

a. Series I - Dihydropyridinium Oximes

The synthetic strategies for the iodo- and bromo-substituted pyridinium oximes 5a, 6a, 5b, and 6b are outlined in Figure 5. Direct iodination of 2-picoline (15) afforded the 3- and 5-iodo-picolines (16 and 17) in roughly equal portions (30% overall). The individual isomers could be separated chromatographically; however, due to the difficulty of separation, the mixture was oxidized with I_2/DMSO to the corresponding aldehydes 18 and 19 (54% overall). Large scale chromatographic separation of the aldehydes was accomplished and the individual aldehydes were condensed with neutral aqueous hydroxylamine to afford the highly insoluble oximes 20 and 21. After experimenting with several alkylating conditions, methyl iodide quaternization to the parent oximes 6a and 5a was accomplished and optimized. The 3-iodo-2-PAM (6a) was the more sensitive compound of the two, and alkylation was best accomplished in methylethylketone, which allowed the product to precipitate from the reaction as it formed. It should be noted that alkylations were performed under nitrogen to suppress disproportionation of methyliodide to iodine and ethane. Gram quantities of these two compounds have been sent to WRAIR for cataloging and further screening. These parent oximes have already been screened (in vitro and in vivo) in our laboratories.

Identical synthetic strategies were employed for the bromo-series. Direct bromination of 15 required sealed vessels, but yields of 22 and 23 were good and pure products were isolated. Reactions in the bromo-series afforded products in similar yields and required the same laborious purifications as seen in the iodo-series. In general the bromo-substituted compounds were more volatile and soluble. Quantities of 5-bromo-2-PAM (5b) have been made and preliminary in vitro data are available. 3-Bromo-2-PAM (6b) awaits the synthesis of larger quantities of oxime 26.

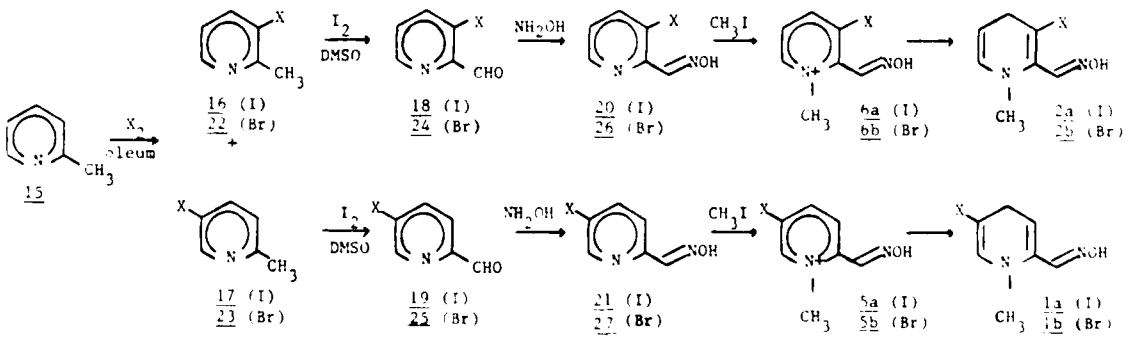


Figure 5. Strategies for Synthesis of 3- or 5-Iodo- and Bromo-Substituted Pyridinium Oximes.

In all cases the methiodide salts (5-6) were converted cleanly to the methochlorides via ion exchange. Reduction chemistry of the iodo-2-PAM's is currently under investigation and involves two basic approaches: hydride and dithionite chemistry.

The syntheses of the 3/5-cyano-2-PAM's 6d and 5d have proved to be difficult. Figure 6 outlines one of the strategies employed. Attempts at direct replacement of the iodo-substituent on aldehydes 18 and 19 afforded low yields and many impurities; therefore, the aldehyde function was protected as the acetal (28 and 29). Thus, while adding a step, it allowed high yield (70-80%) displacement with CuCN to give the cyano-substituted acetals 30 and 31. Deprotection with dilute HCl gave both cyanoaldehydes 32 and 33. 3-Cyano-2-picolinaldehyde (32) proved to be very volatile, as well as easily oxidized. Condensation of the 5-cyano isomer 33 with neutral aqueous hydroxylamine afforded 5-cyano-2-pyridinealdoxime 35. The 3-cyano-isomer 34 appeared to form only as an intermediate, which underwent further cyclization and hydrolysis to compound 36.

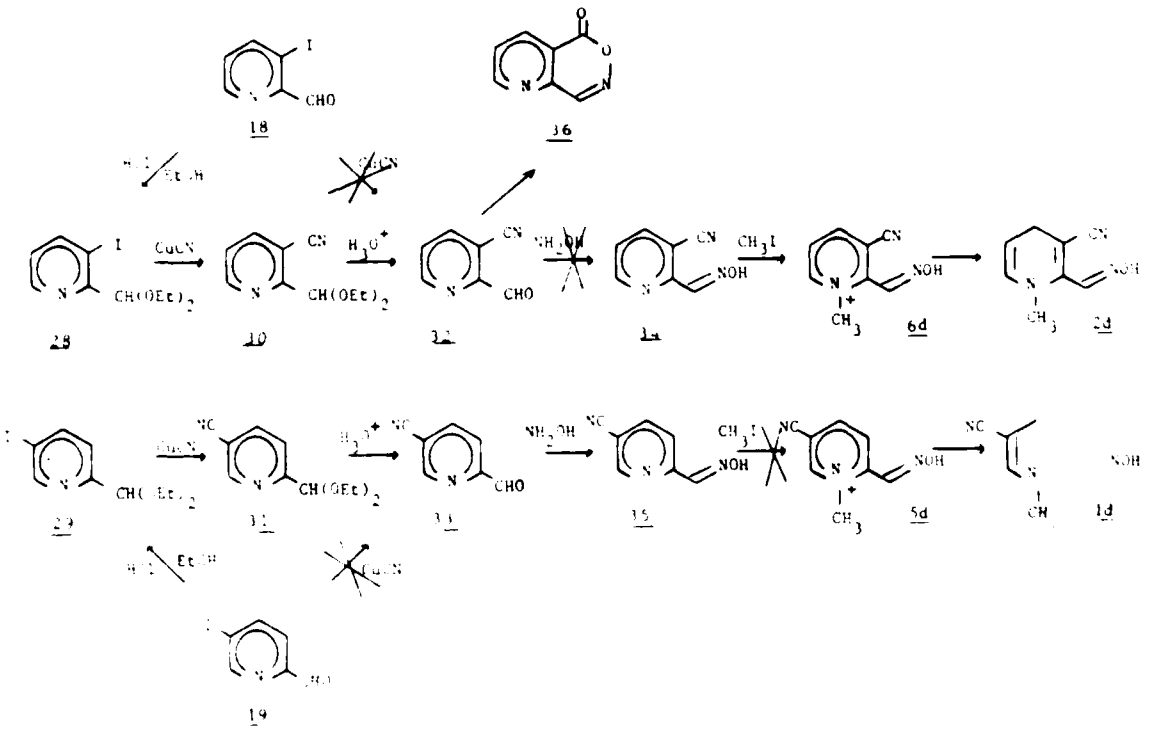


Figure 6. Strategies for Synthesis of 3- or 5-Cyano-Substituted Pyridinium Oximes.

5-Cyano-oxime 35 resisted clean alkylation to 5d under all conditions employed. Alkylation using either dimethylsulfate or methyl iodide required a large number of equivalents of the alkylating agent. The reaction became acidic under the vigorous conditions employed, and the nitrile moiety was repeatedly destroyed. The use of other pyridines as buffers and transalkylation catalysts proved ineffective. We are currently reevaluating the feasibility of synthesizing these compounds.

The nitrile intermediates 30 and 31 did, however, provide us with entry into the carboxamido-substituted series (Figure 7). Controlled basic hydrolysis of the nitriles to carboxamido-acetals 37 and 38 was accomplished in reasonable yields (40-70%) with little purification required. Dilute acid hydrolysis of the acetals afforded 5-carboxamido-2-picolinealdehyde (40) cleanly, however, 39 appears to have formed a bicyclic product, tentatively assigned structure 43. The techniques available to gently hydrolyze 37 to 39 have not yet been exhausted. 5-Carboxamido-2-picolinealdehyde (40) could be carefully condensed with neutral hydroxylamine to afford oxime 42. Quaternization to 5e awaits the synthesis of larger quantities of 42.

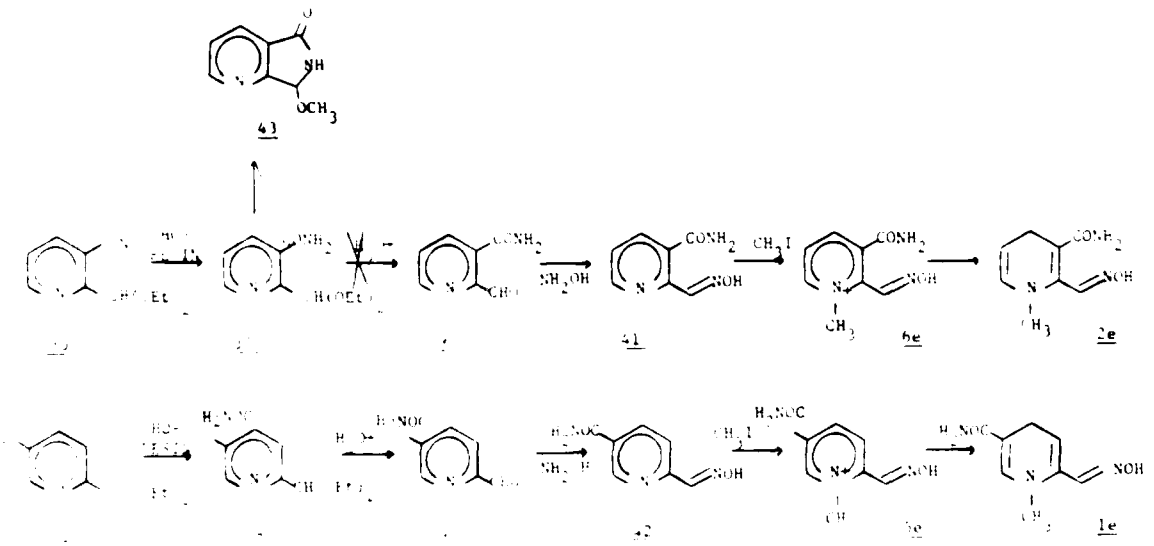


Figure 7. Strategies for Synthesis of 3- or 5-Carboxamido-Substituted Pyridinium Oximes.

Synthesis of 3/5-chloro-2-PAM's 5c and 6c (Figure 8) has been attempted, but preliminary work is not encouraging. Attempts at displacing bromine from 22 and 23 with CuCl did not afford the desired chloropicolines 44 and 45. Displacement of iodine from protected iodopyridinealdehydes 28 and 29, likewise did not appear to give the desired chloroacetals 50 and 51. Cuprous chloride does not appear to be a sufficiently good source of "bare" chloride to displace another halide from the ring; however, we are continuing to search for successful reaction conditions for chlorination.

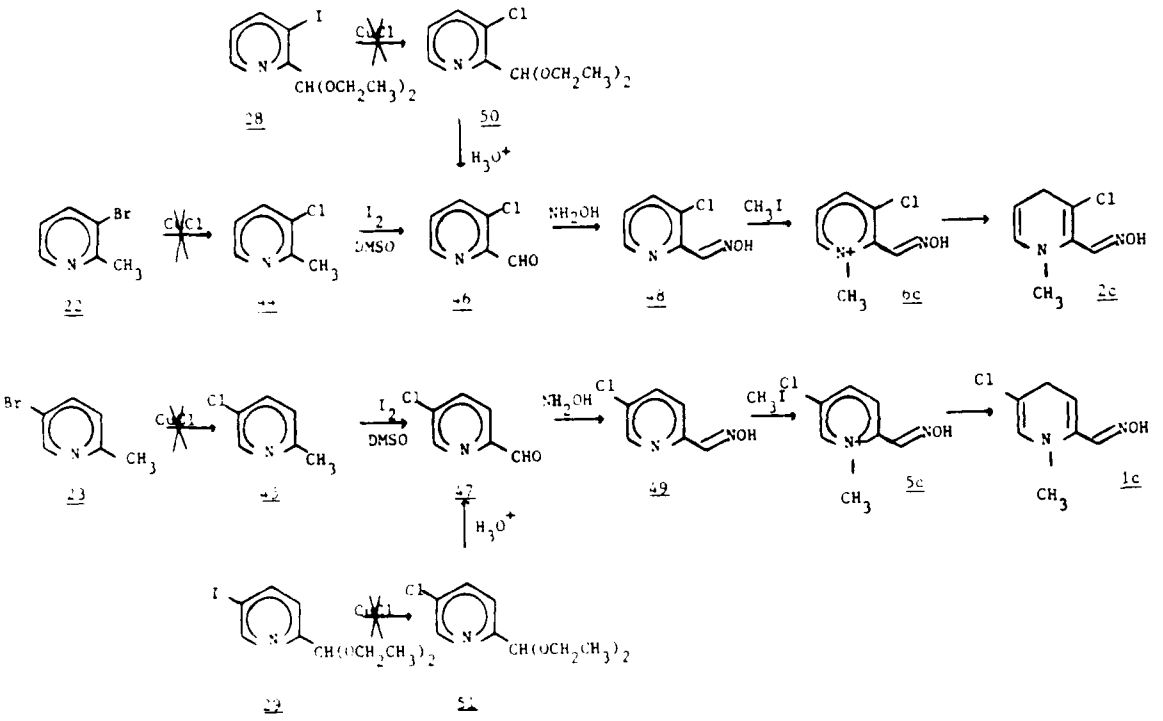


Figure 8. Strategies for Synthesis of 3- or 5-Chloro-Substituted Pyridinium Oximes.

pKa's (10^{-3} M) and partition coefficients (octanol/water and octanol/buffer) have been determined for the parent quaternary oximes, which we had prepared in quantity (6a, 5a, and 5b). Table 1 is a compilation of those data.

Table 1. pKa Values and Partition Coefficients for 3- or 5-Substituted Pyridinium Oximes and 2-PAM.

Compound		pKa	Partition Coefficient P^a	Partition Coefficient P^b
3-I-PAM	6a	7.9	4×10^{-3}	c
5-I-2-PAM	5a	7.5	2×10^{-3}	1×10^{-2}
5-Br-2-PAM	5b	7.6	1×10^{-3}	1×10^{-2}
2-PAM	7	7.9	4×10^{-4}	1×10^{-3}
Pro-2-PAM	4	10.5	5×10^{-3}	4.5×10^{-1}

a) Octanol/water
b) Octanol/0.1 M MOPS pH 7.4
c) Decomposition occurred at pH 7.4

b. Series II - Tetrahydropyridinium Oximes

Figure 9 outlines the scheme used to synthesize the doubly latent compounds 3a-c. Following the literature preparation outlined by Shek et al.[21] and starting from pyridine-2-carbaldoxime (51), the desired cyanide addition product 3a was obtained. The yield was considerably less than that reported by Shek et al.[21] As a solid, prodrug 3a was not stable at room temperature. It was reasonably stable at room temperature stored under nitrogen, but darkened considerably with time. Substitution of KSCN or KBr for KCN under identical reaction conditions (pH 1-2) did not yield the desired products 3b and 3c. The desired products 3b and 3c (X=SCN,Br) were isolated when the pH of the reaction was brought to the 7-8 range. A 40-60% yield was obtained and the desired products were characterized spectrally.

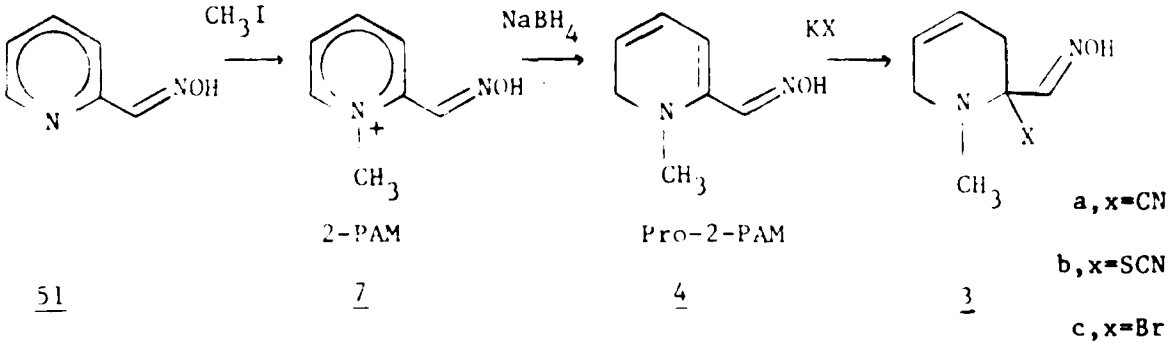


Figure 9. Strategies for Synthesis of Dihydro- and Tetrahydro-Pyridinium Oxime.

The prodrug forms 3a-c were monitored for conversion to 2-PAM in phosphate buffer (150 mM, pH 6.5-8.5). The progress of the conversion was monitored with time via HPLC. In this manner 2-PAM could be positively identified and quantitated by UV detection. The only compound which showed significant conversion to 2-PAM was 3a (X=CN), but conversion was only on the order of 20%. Prodrug 3b exhibited no measurable conversion to 2-PAM and the bromo-derivative 3c showed only a slight (5-10%) conversion. We are currently reexamining this approach.

2. Bioassay

a. In Vitro

Regenerators of AChE were evaluated in vitro, using the assay developed in our laboratory, which incorporated immobilized enzyme and the Ellman technique.[28] Eel AChE was attached via a stable Schiff base link to functionalized polyethylene beads using a modification of the procedure of Ngo, Lardler and Yam as outlined in Figure 10.

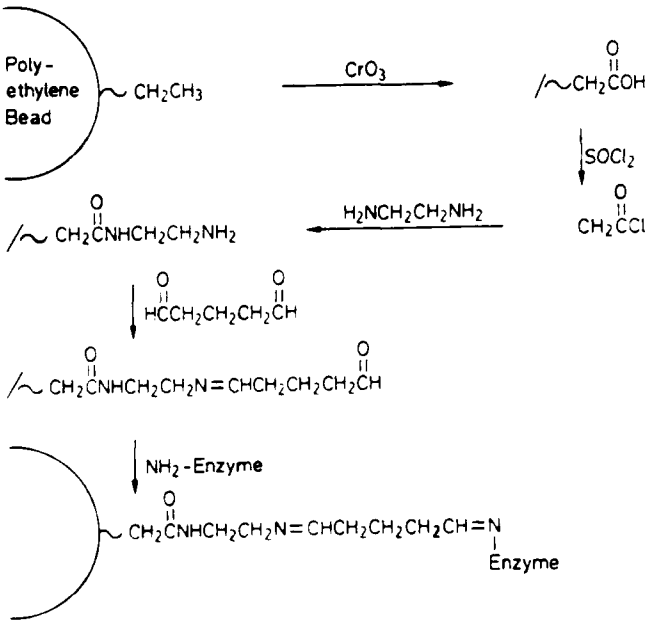


Figure 10. General Scheme for Immobilization of AChE on Polyethylene Beads.

Utilizing acetylthiocholine as substrate and DTNB as chromogen, the hydrolysis was followed spectrophotometrically at 412 nm. A flow-through apparatus powered by a peristaltic pump was assembled (Figure 11), which allowed sequential and stepwise: (1) determination of initial immobilized enzyme activity, (2) inhibition with organophosphate, (3) reactivation with various oxime concentrations, (4) determination of regenerated enzyme activity, and (5) washing between steps to remove residuals.

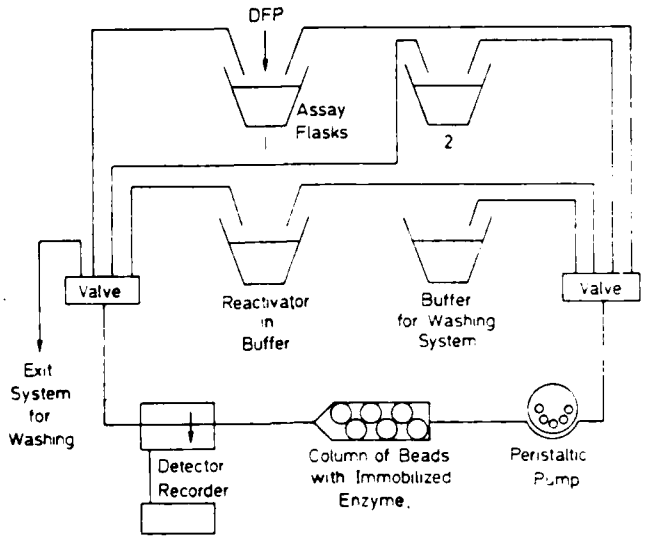
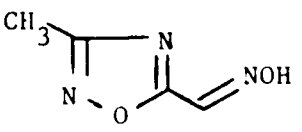


Figure 11. Schematic of Closed Loop, Flow-Through System for Immobilized Acetylcholinesterase Assay.
Flasks 1 and 2 contain substrate (ATC, 10^{-3}M) and chromogen (DTNB, 5 X 10^{-4}M) in MOPS buffer (0.1 M, pH 7.8) at 37°C. The peristaltic pump was run at a flow rate of 5.8 ml/minute and the detector was set at 412 nm.

The immobilized enzyme retained activity from 4-8 months when stored at -16° C. DFP-inhibited AChE could be regenerated using 2-PAM (7), TMB-4 (8) and MINA (10) as shown in Figures 12-14.

New regenerators synthesized in our laboratories (3-iodo-2-PAM (6a), 5-iodo-2-PAM (5a) and 5-bromo-2-PAM (5b), as well as one synthesized at SRI (KHB-0024E [53]) were evaluated in vitro as shown in Figures 15 and 16. In all cases the regenerating activities found for these compounds were less than that shown by 2-PAM. All of the reactivation curves exhibited the same basic shape as well as dose-response (extent of reactivation) phenomenon.



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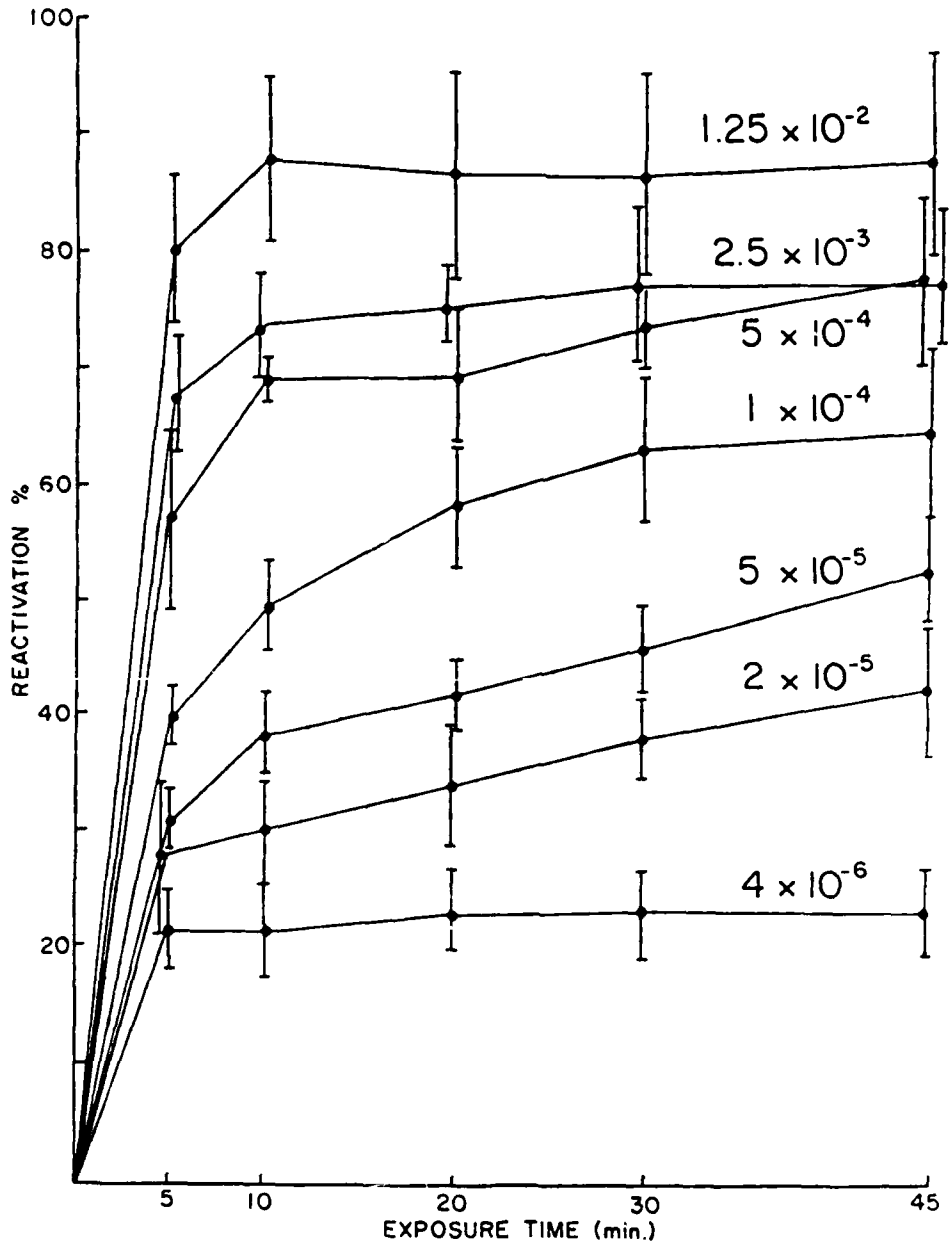


Figure 12. Effects of 2-PAM on Regeneration of Immobilized Eel AChE.

Concentrations of oxime expressed in molarity. The Enzyme was inhibited with DFP (6 X 10^{-4}M), then washed free of excess DFP. Enzyme was then exposed to varying concentrations of 2-PAM for time points shown. After each exposure to 2-PAM, the enzyme was washed free of regenerator, esterase activity measured, and exposure to regenerator resumed. These steps were repeated until the enzyme had been exposed to regenerator for a total of 45 minutes.

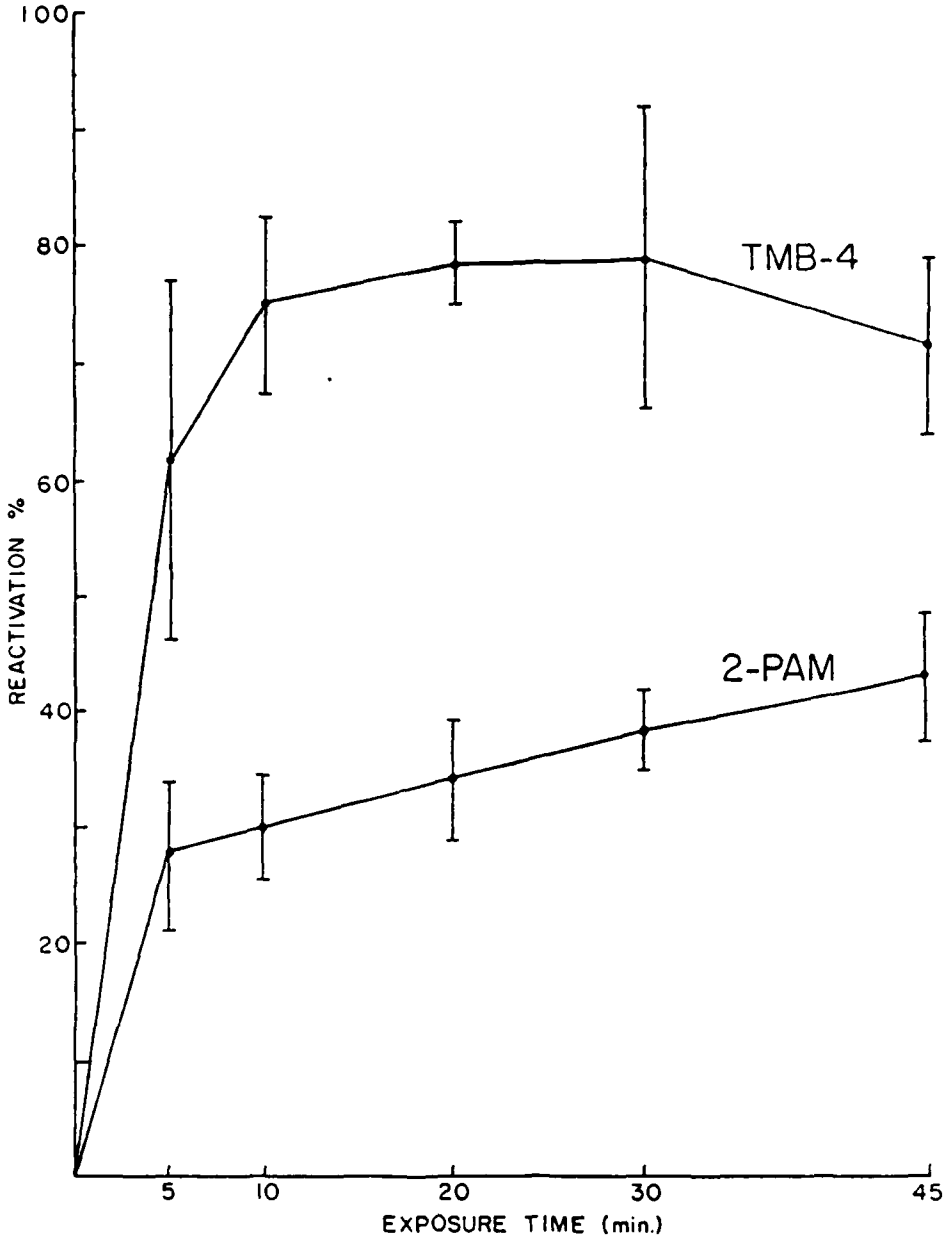


Figure 13. 2-PAM vs. TMB-4 as Regenerator of DFP-Inhibited Immobilized Eel AChE.

Concentrations were 2 X 10^{-5}M. Conditions as described in Figure 12.

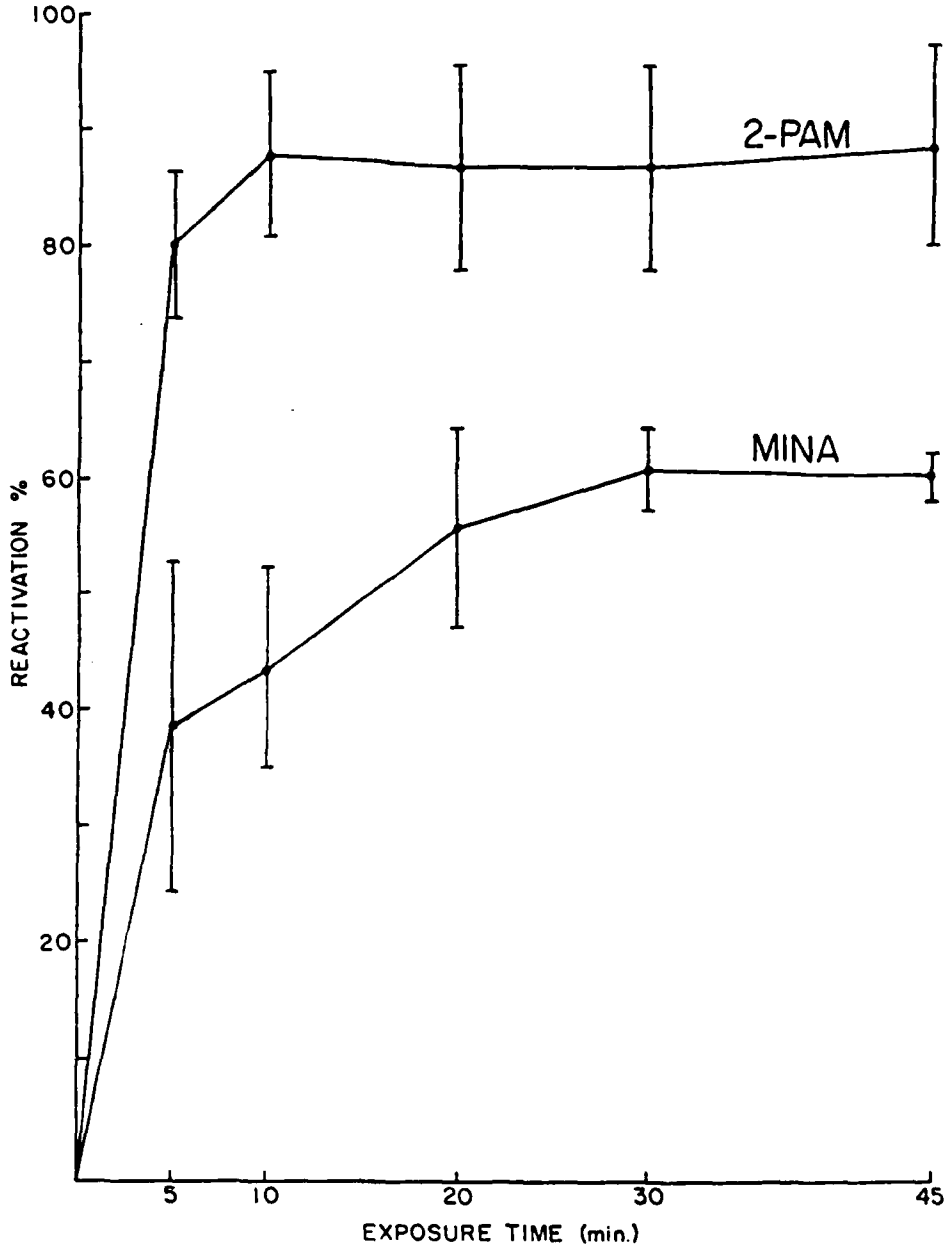


Figure 14. 2-PAM vs. MINA as Regenerator of DFP-Inhibited Immobilized Eel AChE.

Concentrations were 1.25×10^{-2}M. Conditions as described in Figure 12.

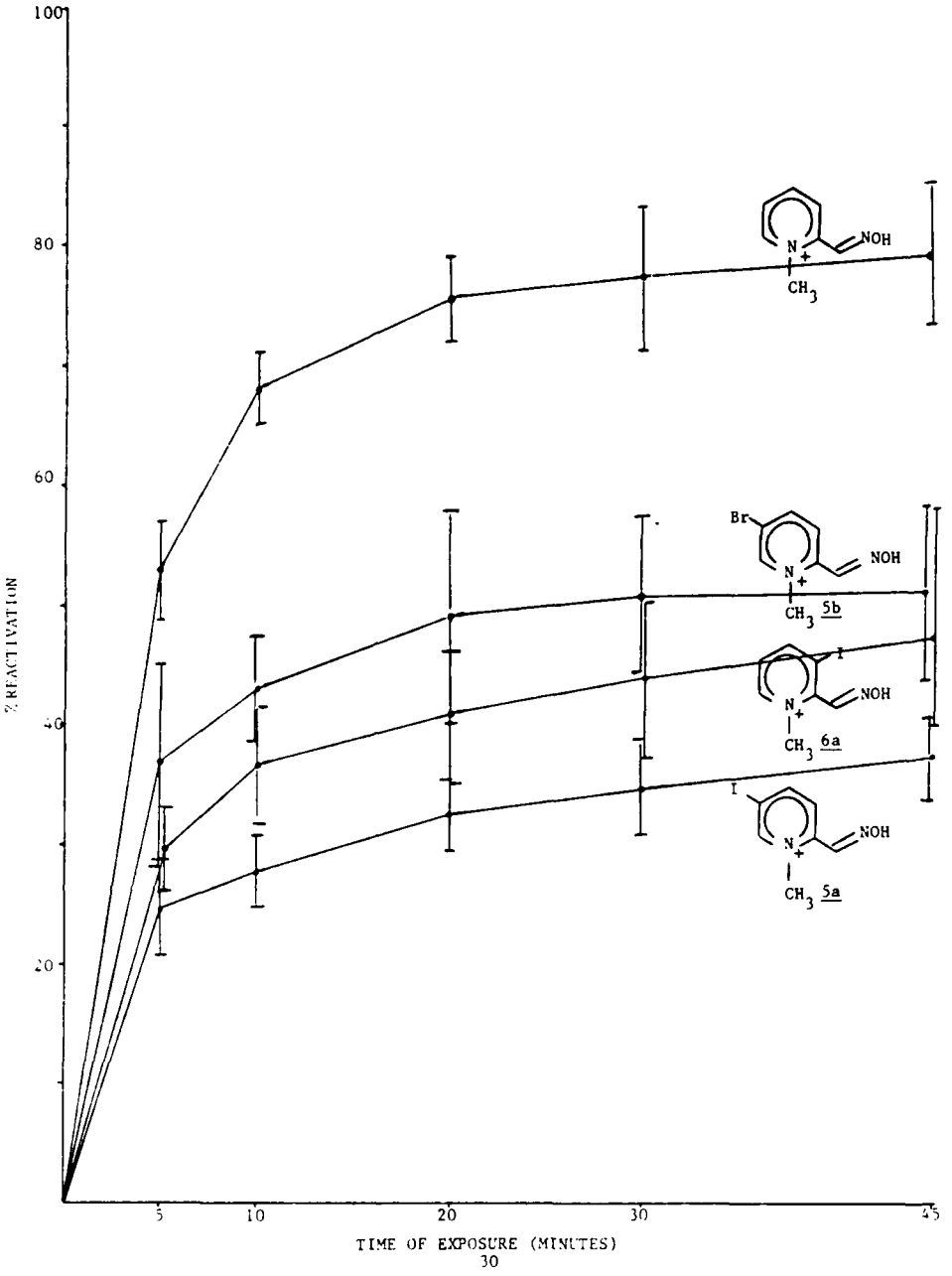


Figure 15. Various 3/5-Substituted-2-PAM's vs. 2-PAM as Regenerators of DFP-Inhibited Immobilized AChE.

Concentrations were 1 X 10^{-3}M. Conditions as described in Figure 12.

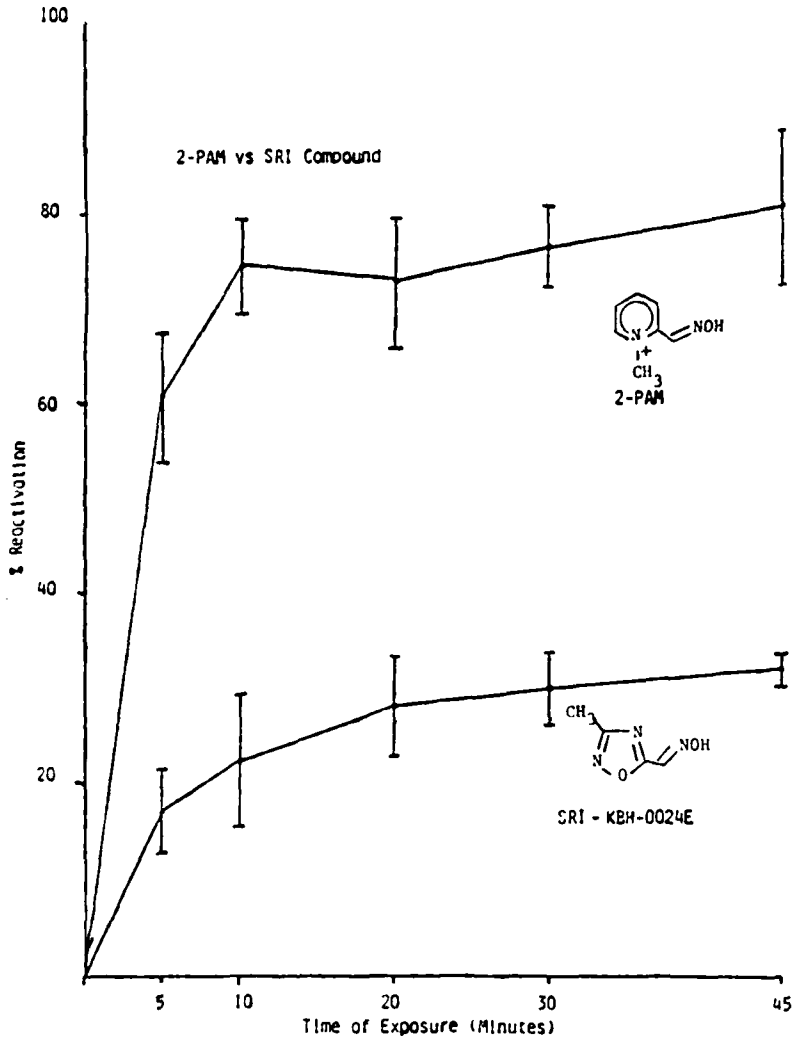


Figure 16. An Oxadiazole Oxime vs. 2-PAM as Regenerator of DFP-Inhibited Immobilized Eel AChE.

Concentrations were 1 X 10^{-3} M. Conditions as described in Figure 12.

These observations led us to the examination of the inhibition curves of the enzyme - the inverse of the reactivation process. Inhibition of immobilized eel AChE with various concentrations of DFP (see Figure 17) resulted in curves which were virtual mirror images of the reactivation curves (Figure 12). We extended this investigation to soluble enzymes (nonimmobilized), such as purified eel AChE and AChE from crude mouse brain homogenate, and found the same characteristic inactivation curves (Figures 18 and 19).

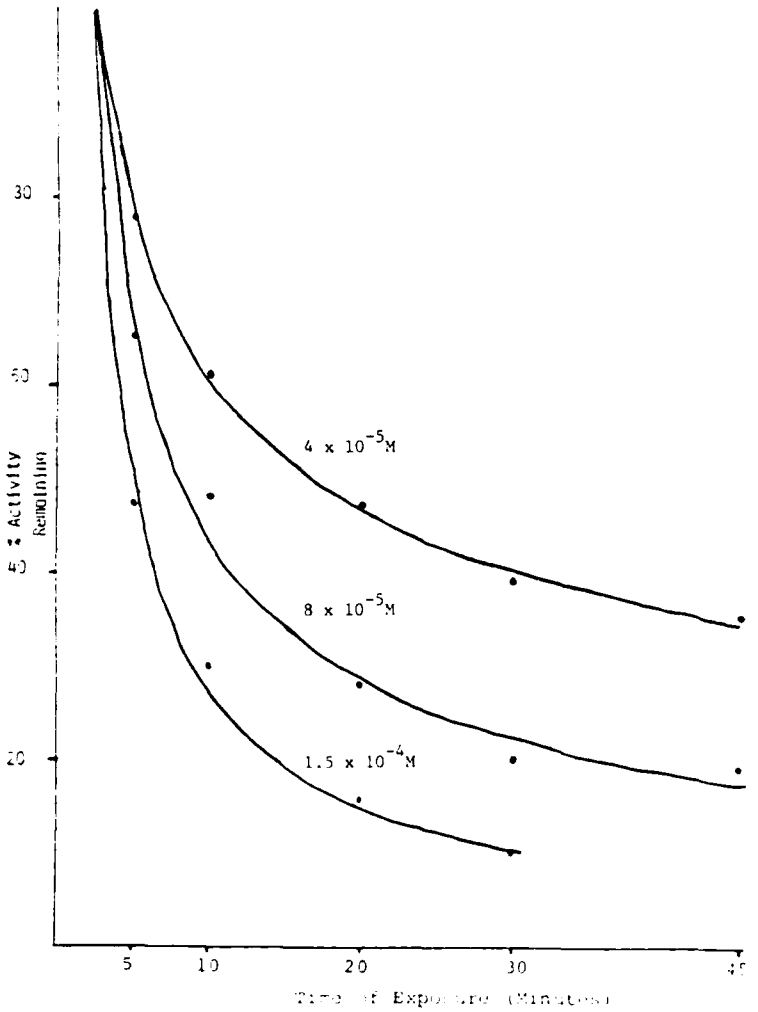


Figure 17. Effects of DFP on Inhibition of Immobilized Eel AChE. Concentrations of DFP in molarity. Enzyme was exposed to varying concentrations of DFP for the time points shown. After each exposure to DFP, the enzyme was washed free of inhibitor, and the remaining esterase activity measured. A fresh set of immobilized AChE was used for each time point.

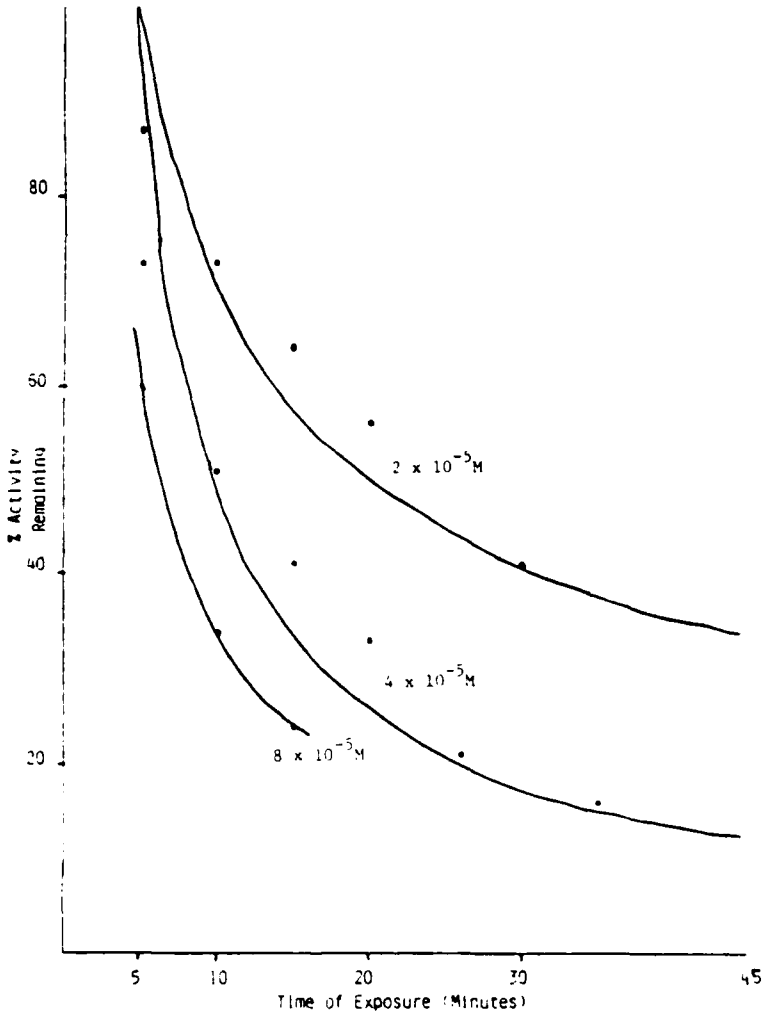


Figure 18. Effects of DFP on Inhibition of Soluble Purified Eel AChE.

Concentrations of DFP in molarity. An aliquot of soluble enzyme was exposed to varying concentrations of DFP for the time points shown. After exposure to a given concentration of DFP for a given time, an enzyme activity measurement was taken. A fresh aliquot of AChE was used for each time point and DFP concentration.

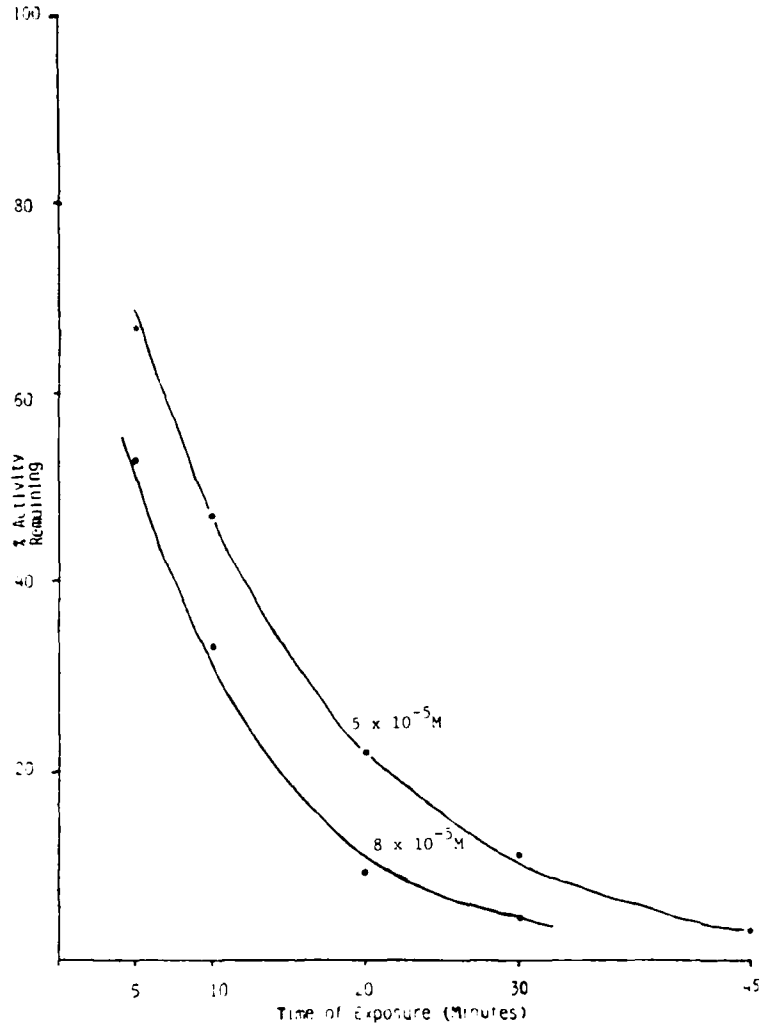
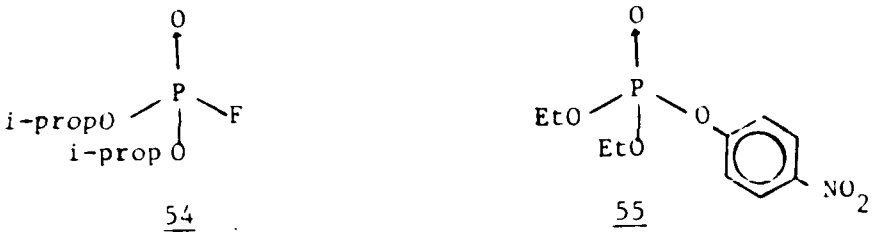


Figure 19. Effects of DFP on Inhibition of Mouse Brain AChE.

Concentrations of DFP in molarity. A fresh mouse brain was homogenized and an aliquot of the homogenate was exposed to varying concentrations of DFP for the time points shown. After exposure to a given concentration of DFP for a given time period, an enzyme activity measurement was taken. A fresh aliquot of homogenate was used for each time point and DFP concentration.

As illustrated in Figure 17, the immobilized AChE has the potential to be used for studying the effects of organophosphates. Therefore, we examined the inhibitory effects of another organophosphate, diethyl-p-nitrophenylphosphate (paraoxon or DNP). Again using immobilized eel AChE, the enzyme was exposed to a series of concentrations of either DFP (54) or DNP (55) for ten minutes and washed free of inhibitor, and the residual activity was measured. Figure 20 shows that a linear relationship exists between log dose of organophosphate and enzyme activity. DNP was an order of magnitude more active than DFP.



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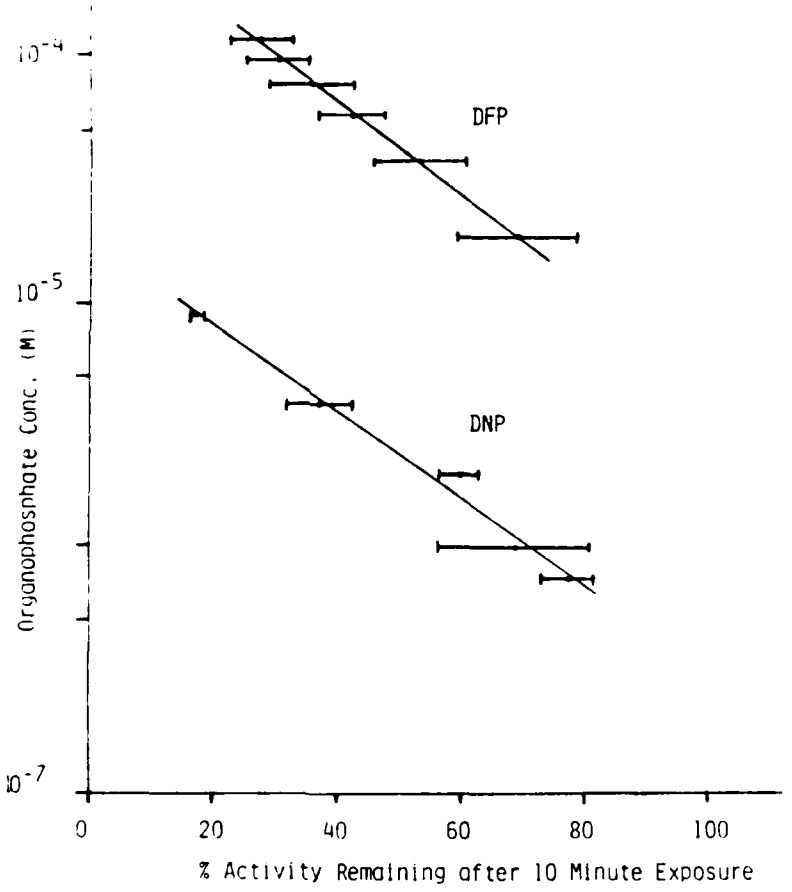

Figure 20. Effects of DFP and DNP on Immobilized Eel AChE.

Concentrations expressed in molarity. Enzyme was exposed to a concentration of DFP or DNP for about 10 minutes, then washed free of any remaining inhibitors. The residual esterase activity was then measured. Each point represents a fresh portion of immobilized enzyme exposed to a given concentration of DFP or DNP.

In an attempt to answer whether immobilized enzyme is a valid model for the enzyme in a biological system, fresh brain slices were used in place of beads containing immobilized enzyme. Native AChE exposed on the surface of the tissue slices could be inhibited with DFP and regenerated with 2-PAM. Figure 21 illustrates that AChE bound in a real biomatrix demonstrates virtually identical curves to those seen with enzyme immobilized on polyethylene beads (Figure 12).

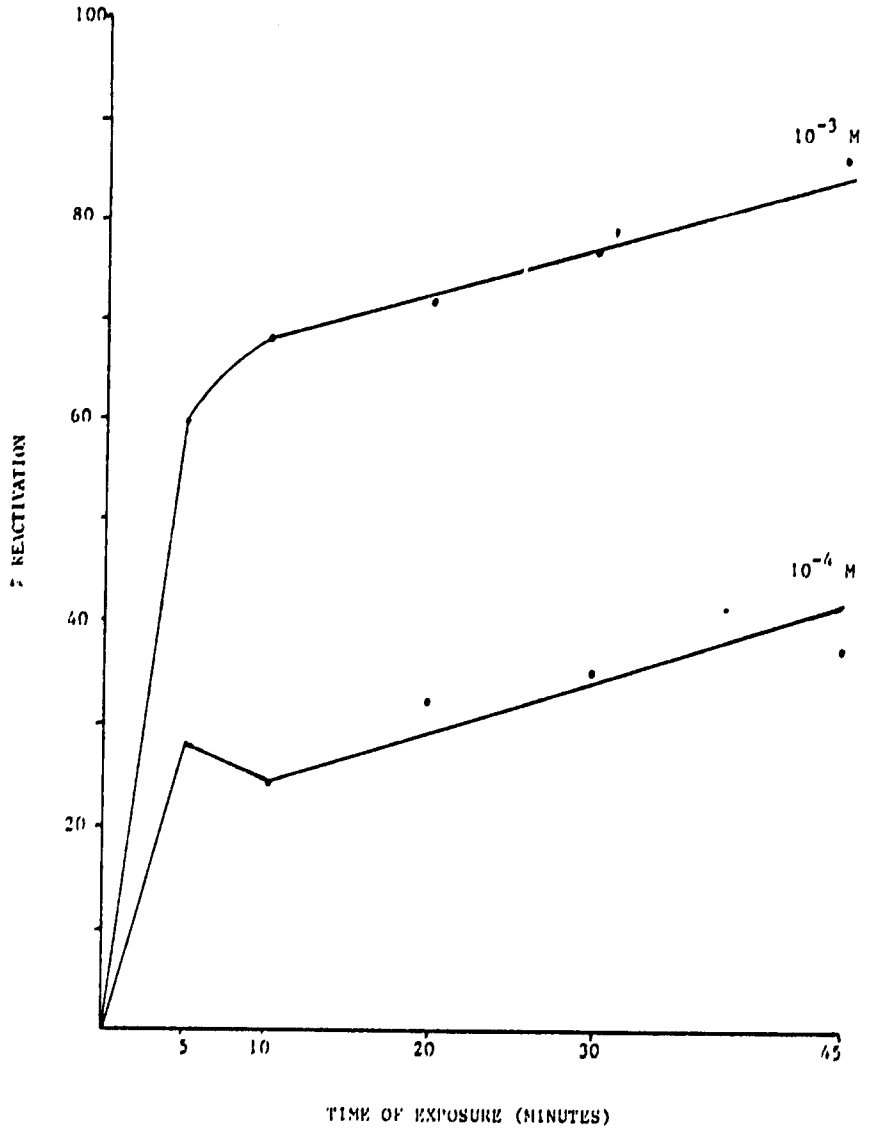

Figure 21. Effects of 2-PAM on Regeneration of Mouse Whole Brain AChE.

Concentrations of 2-PAM expressed in molarity. Using the AChE assay technique outlined in Figure 12, slices of freshly sacrificed mouse brain were used in place of immobilized enzyme containing beads. The intact tissue was assayed for AChE activity, inhibited with DFP, washed, then exposed to 2-PAM for the time points shown. The tissue was then washed again to remove excess regenerator and assayed for AChE activity. Each point represents the mean of 3 mice for 10^{-3}M 2-PAM experiments and 2 mice for 10^{-4}M experiments.

b. In Vivo

Preliminary determinations of ED_{50} values for regenerators synthesized in our laboratories have been performed. Following $2xLD_{50}$ DFP (SC) challenge, mice were immediately injected intramuscularly with 2-PAM (7), 3-I-2-PAM (6a) and 5-I-2-PAM (5a). The results in Table 2 show that 5-I-2-PAM (5a) was significantly more effective than 2-PAM (7) in saving mice from DFP intoxication.

Table 2. ED_{50} Values for 3- or 5-Substituted Pyridinium Oximes and 2-PAM in DFP Challenged Mice

Compound	ED_{50} (mg/kg)
2-PAM (7)	11-12
Pro-2-PAM (4)[a]	21-22
3-I-2-PAM (6a)	8-9
5-I-2-PAM (5a)	0.3-0.4

DFP ($2xLD_{50}$, sc, water), oxime (IM, water, 1 ml/kg inj vol)
[a]IV, 50 mM citrate, pH 3.5

Brain levels of AChE in mice intoxicated with DFP were examined. Figures 22-25 show the results found when mice were injected with a dose of DFP (2 mg/kg or 4 mg/kg or 6 mg/kg), then sacrificed at either 3 or 5 minutes, and the brain (or brain section) was assayed for AChE activity (Ellman technique).[23] These figures illustrate that at 4 and 6 mg/kg ($2xLD_{50}$) there is a marked decrease in AChE activity level at 3 or 5 minutes after injection and at death. At a 2 mg/kg dose, there is a depression of AChE activity in the brain 3 minute postchallenge followed by a slight rebound at 5 minutes. Figure 25 illustrates how AChE activity in the entire brain drops off precipitously following a lethal dose of DFP at 3 and 5 minutes as well as at death. Administration of 2-PAM (30 mg/kg) does not markedly regenerate CNS AChE activity despite the fact that animals do not die (see 10 min time point).

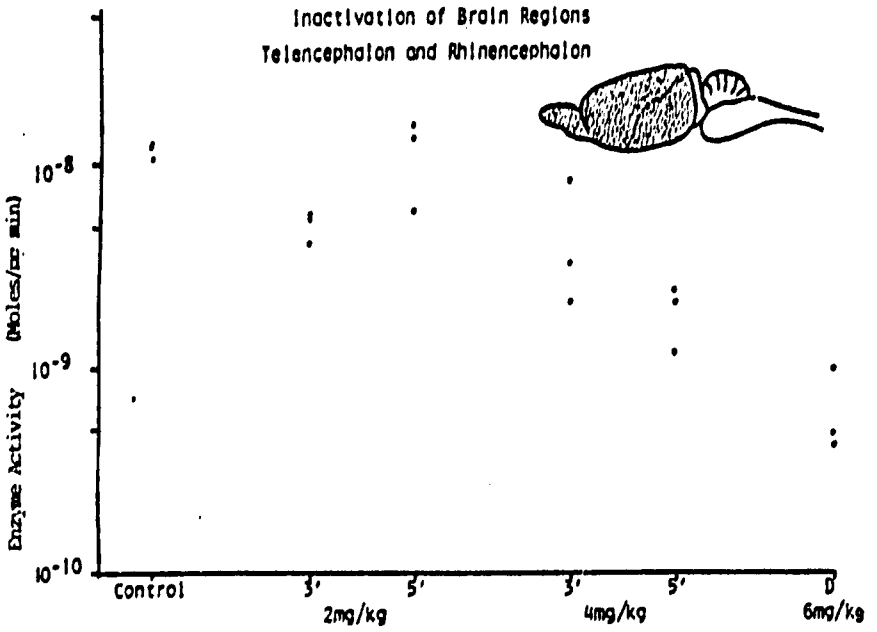

Figure 22. Effects of DFP on AChE Activity in Forebrain of Mice.

Mice were injected with 2, 4 or 6 mg/kg DFP (sc). At 3 and 5 minutes post-injection, as well as at death, the brain was removed and a section assayed for AChE activity. Each point represents one animal.

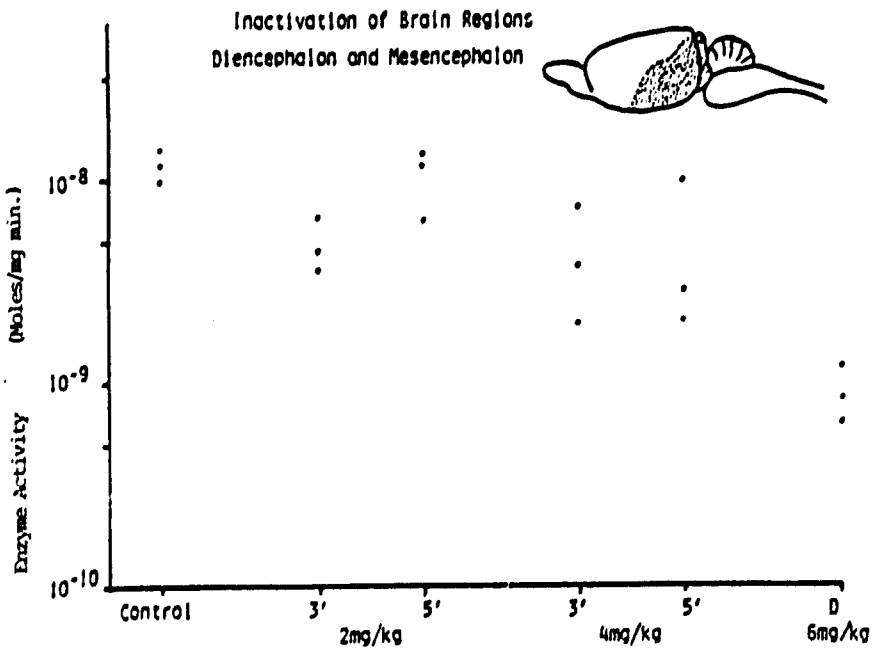

Figure 23. Effects of DFP on AChE Activity in Midbrain of Mice.

Conditions as described in Figure 22.

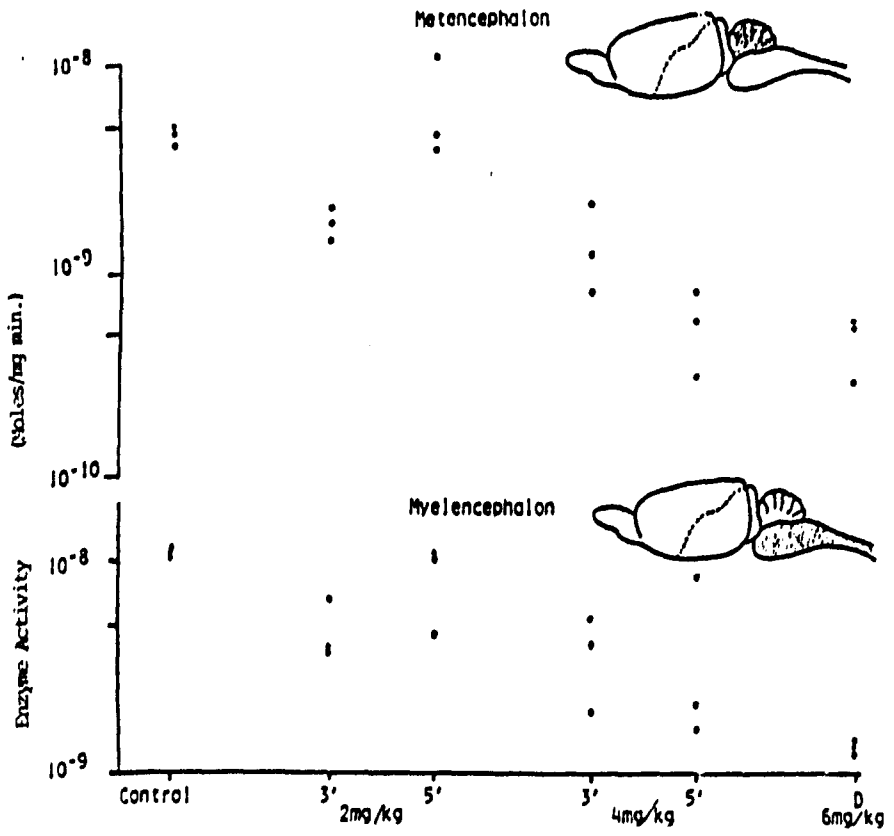

Figure 24. Effects of DFP on AChE Activity in Hindbrain and Brainstem of Mice.

Conditions as described in 22.

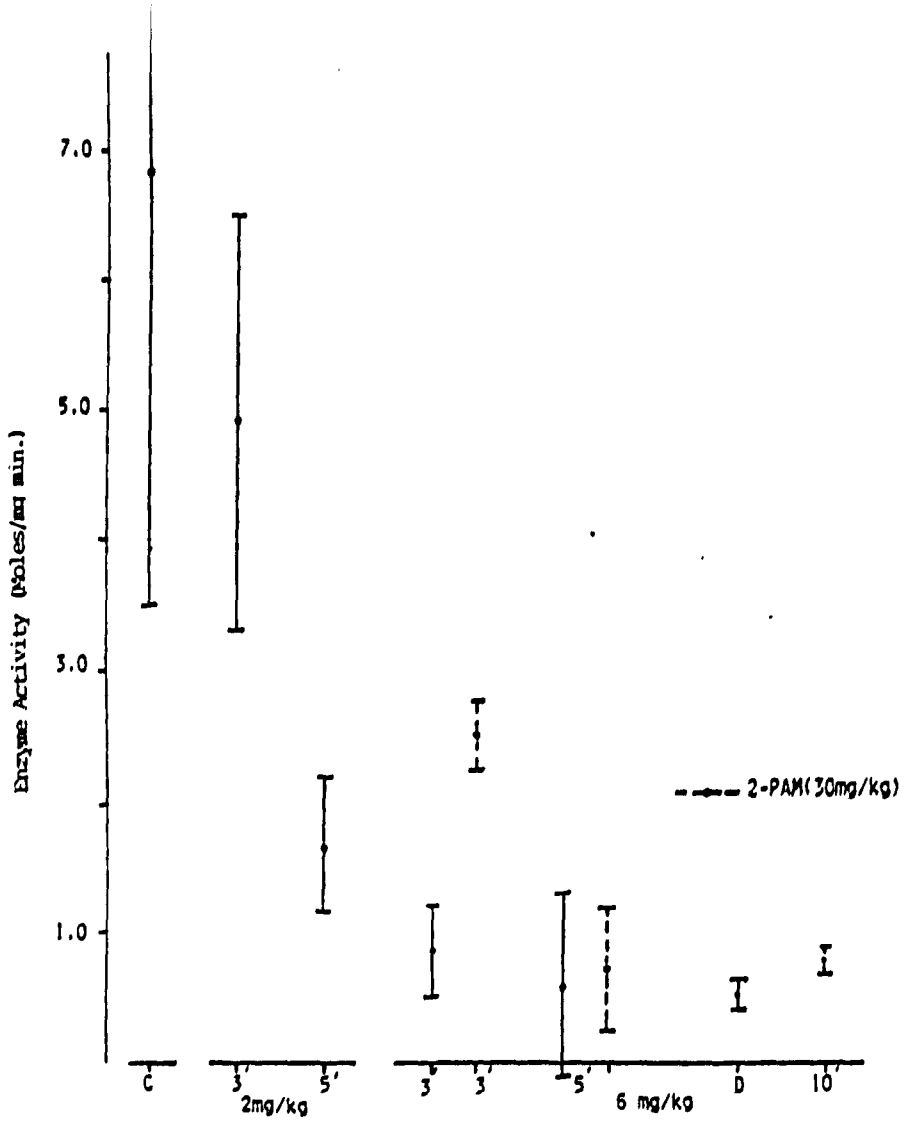

Figure 25. Effects of DFP on AChE Activity in Mouse Brain.

Mice (3-6 per group) were injected with either 2 or 6 mg/kg (2 x LD_{50}) of DFP (sc). Animals challenged with 6 mg/kg DFP also received 30 mg/kg of 2-PAM (im) approximately 10 seconds later. At 3, 5 or 10 minutes, as well as at death (D), the brain was removed and assayed for AChE activity.

Extensive investigations were undertaken into nonradiotracer techniques for monitoring the biodistribution and elimination of pyridinium oximes. The focus has been on the use of high performance liquid chromatography due to its flexibility. Reversed-phase separations using a variety of column packing (silica and hydrocarbon based) and elution conditions were examined. Silica based C8 and C18 bonded phases proved effective at separating pyridinium oximes when eluted with ion-pairing reagents. However, the most flexible system was found to be based on a hydrocarbon-resin spherical column packing. This neutral hydrophobic material could tolerate a wide range of pHs (1-13), which allowed elution with aqueous acetonitrile or aqueous methanolic mobile phases from pH 3.5-10.5. The advantage of the system stemmed from the fact that each pyridinium oxime regenerator has a discrete λ_{max} which is pH dependent. 2-PAM, as an example, has an intense absorption at pH 9-10 (335 nm) with an extinction coefficient (19,000) almost twice that shown at pH 3-4 (295 nm, 12,000). This increase in sensitivity was desirable, but conditions required to achieve it could not be used with silica-based reversed-phase columns due to their base instability. Figure 26 illustrates a typical separation on the system at pH 10.5 (0.1 M Na_2CO_3) and monitored at 335 nm. Biological components remaining in prepared tissue extracts do not interfere. When the variable wavelength LC detector is coupled with a recording integrator, the system was used to quantitatively identify and determine the *in vitro* conversions of prodrugs to their parent pyridinium oximes as well as *in vivo* tissue distribution. Figure 27 shows distribution and elimination patterns of 2-PAM in selected tissues - including the brain. Figure 28 illustrates how Pro-2-PAM increases brain levels an order of magnitude above those found for 2-PAM. 5-I-2-PAM (5a), however, could not be measured in the brain at the doses administered (50 mg/kg).

E. Discussion

1. Chemistry

Some of the intended synthetic approaches have been either abandoned or revised because the procedures were cumbersome and separation and purification of the resulting products were laborious and time consuming. However, the synthetic schemes developed (Figures 5-8) have produced the 3/5-I-2-PAM's (6a and 5a) and 3/5-Br-2-PAM's (6b and 5b). Low yields and difficult chromatographic purifications have caused problems in the synthesis of gram scale quantities of these compounds. We have requested large quantities of intermediates synthesized by the preparations laboratories (arranged by H. A. Musallam). Our goal is to prepare roughly 10 g of each of the above parent quaternary oximes so that they can be extensively screened *in vitro/vivo* and biodistribution data can be collected. In addition, we must retain sufficient material to develop the dihydro-prodrug forms 1a, 1b, 2a and 2b. The prodrug forms will require analysis of physical and spectral properties and bioassay data for comparison with parent compounds.

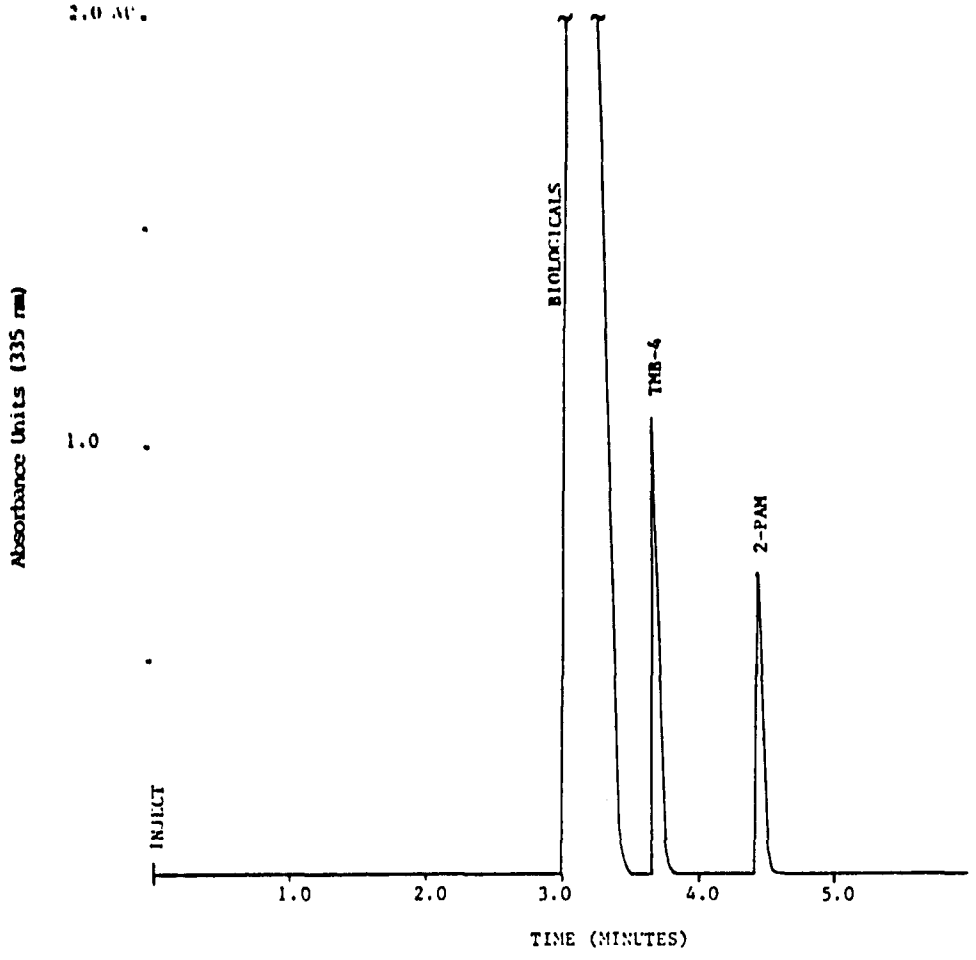

Figure 26. Separation by HPLC of Some Pyridinium Aldoximes.

A 20 µl portion of tissue extract, prepared as outlined in the experimental section, was injected on column. At ambient room temperature the sample was separated, using a 0.5 x 25 cm PRP-1 10 µm column eluted with 0.1 M Na_2CO_3 (pH 10.5) at 1 ml/minute and detection at 335 nm.

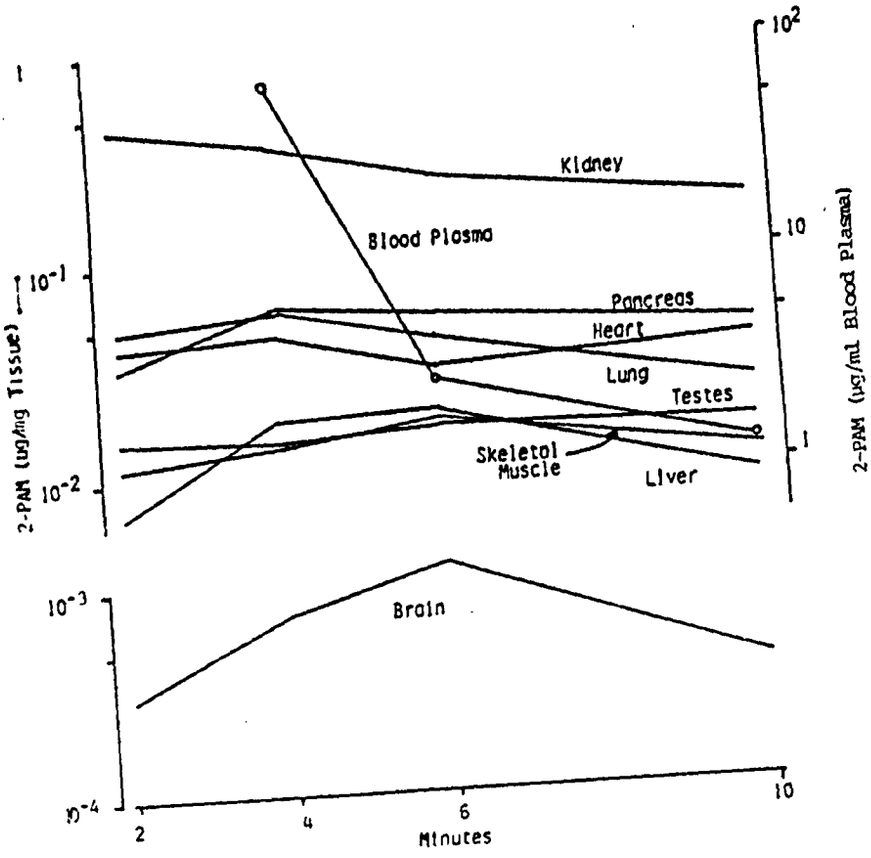

Figure 27. Tissue Distribution of 2-PAM in Mice by HPLC Analysis.

Tissue levels of 2-PAM were measured following im injection (100 mg/kg) in mice. Each point represents one animal. The animals were sacrificed at the time points shown and the tissue treated as outlined in the experimental section. A 20 µl portion of tissue extract was injected on column. HPLC analysis was as outlined in Figure 24.

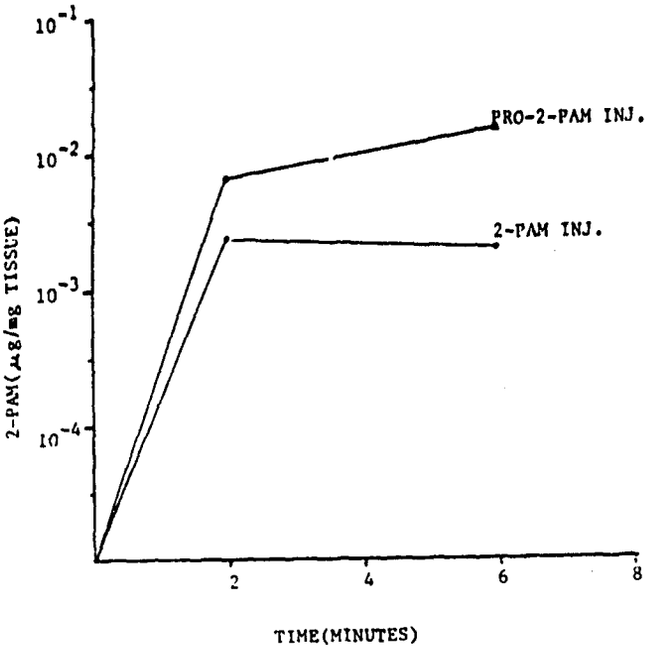

Figure 28. Brain Levels of 2-PAM in Animals Injected with 2-PAM vs. Pro-2-PAM.

Brain levels of 2-PAM were measured after either im (hindlimb) injection (100 mg/kg) of 2-PAM or iv (tail) injection (100 mg/kg) of pro-2-PAM in mice. Each time point represents the mean of 5 mice (except for pro-2-PAM at 10 minutes, which was 3 mice). Mice were sacrificed at the time points shown and brain tissue was treated as outlined in the experimental section. A 20 μl portion of prepared tissue extract was injected on column and analyzed, using the HPLC conditions outlined in Figure 24.

Synthesis of the 3/5-Cl-2-PAM's (6c and 5c) has been initiated. Although preliminary work on and initial steps in the scheme are not encouraging, we are by no means dismissing the idea of making the chloro-2-PAM's. Direct chlorination of picoline (15) does not appear to be a valid approach as was the case for the iodo- and bromo-series (Figure 5). However, we are confident that chloride displacement of an iodo- or bromo-intermediate can be accomplished.

The 3/5-cyano-2-PAM's (6d and 5d) have been the biggest disappointment to date. The nitrile substituent is documented to have the greatest effect on the stability of dihydropyridines[35] and coupled with its rather small size makes these target compounds highly desirable. However, the close proximity of a 3-CN substituent to functional groups at position 2 invites easy intramolecular interactions. A 5-cyano moiety, while far enough away from the 2-position, deactivates the ring sufficiently to prohibit facile quaternization. Efforts have not been abandoned, but prospects are not encouraging.

Prospects for the 3/5-carboxamido-2-PAM's 6e and 5e look good. We feel that the carboxamido function, while bulkier than a cyano group, is not as reactive and will, with effort, allow synthesis of the desired parent quaternary oximes.

It should be noted here that 3-I-2-PAM (6a) proved to be unstable in aqueous solutions at physiologic or basic pH's. The observation was disturbing, but not unexpected. It does point to the general difficulties involved with 2,3-isomers. The close proximity of a nucleophilic oxime moiety at the 2-position to a labile or reactive substituent at the 3-position can be disastrous chemically. If similar problems are seen with 3-bromo-2-PAM (6b), we may reevaluate the desirability of making 3-substituted-2-PAM's. Our intent for now, however, is to synthesize as many of the 2,3-isomers as possible, which would allow us to draw some conclusions about steric versus electronic effects on the 2-PAM molecule when binding and reactivating capabilities are examined.

The observed pKa values for the halogen substituted 2-PAM's were consistent with expectations - electron withdrawal made the oximes slightly more acidic yet within the acceptable range of 7-8. Partition coefficient data showed halogen substitution to improve octanol solubility, though not to the magnitude that pro-2-PAM does (see Table 1).

Work on the doubly latent species 3 has not progressed as envisioned. The synthesis of the tetrahydro-pyridinium oximes X=SCN and Br required more basic conditions than that where X=CN. This indicated that conversion of these prodrug forms to 2-PAM might require different conditions as well. When conversions from prodrug form 3 to 2-PAM were measured in 150 mM phosphate buffer, X=CN afforded a 20% conversion, while X=SCN and Br fared far worse. The conversions were measured over a pH range of 6.5-8.5, and nowhere did we even approach the desired 80-100% conversion rates required for generating effective levels of 2-PAM. The search for an ideal X will continue, but it will not be allowed to monopolize time and effort.

2. Bioassay

a. In Vitro

The development of the immobilized enzyme screening assay was the culmination of many attempts to make a soluble AChE assay workable. The soluble AChE assay had many drawbacks including competition of both enzyme and regenerator for substrate and the problem of excess organophosphate. 2-PAM is known to react directly with organophosphates in solution to generate phosphorylated 2-PAM, which is itself a potent deactivator of AChE. In addition, phosphorylated 2-PAM was generated in the reactivation process. This pool of excess organophosphate and phosphorylated regenerator caused uncertainty in the soluble AChE assay.

The immobilized AChE assay system developed in our laboratory allows for the independent and sequential determination of initial enzyme activity, enzyme inactivation, enzyme regeneration and determination of regenerated enzyme activity. By washing the immobilized AChE between steps, one can remove excess DFP, oxime, phosphorylated oxime, acetylthiocholine and DTNB, thus eliminating undesirable side reactions. The DFP-inactivated AChE spontaneously regenerates to about 10-15% of the initial enzyme activity. The amount of aging of the DFP-inactivated enzyme was negligible, as was the loss of enzyme activity due to enzyme degradation during the course of a 2 hour experiment.

Problems with bead to bead variations were overcome to a certain extent with the acquisition of precision ground 4 mm polyethylene spheres. In addition, the use of overhead motor driven propeller stirring provided a gentler mixing of bead suspensions, afforded less surface damage and gave more uniform chemically reacted beads. Immobilized eel AChE activity averaged 0.4 unit/bead.

The assay was effective for the determination of the regenerating capabilities of quaternary pyridinium oximes (e.g., 2-PAM and TMB-4) as well as an uncharged oxime (MINA). The order of regenerating ability was TMB-4 > 2-PAM > MINA, which was consistent with earlier observations.[8,27] There was, at all concentrations of regenerators employed, an initial rapid recovery of enzyme activity, which was followed by a slower drift to higher enzyme activity. The first 5 minutes of exposure to regenerator produced a majority of the regenerated activity observed at 45 minutes. This phenomenon was reported earlier and was attributed to a rapid initial equilibrium followed by a slow drift to higher activity due to the breakdown of phosphorylated oxime.[37] Since our system allowed for the removal of any phosphorylated oxime, we do not feel this is a valid explanation for our experimental results. The question of why lower doses of regenerators don't eventually reactive to the same level as higher doses was puzzling as well. The implication is that there may be populations of enzyme which have different sensitivities to both inactivation and reactivation. This encouraged us to approach the problem from the inverse, i.e., inactivation experiments. Figures 17-19 illustrate that inactivation characteristics (of either immobilized or soluble enzyme) closely parallel those of the reactivation process.

One conclusion which can be drawn is that immobilization does not appear to affect the accessibility of the enzyme active site to inhibitor or substrate. As yet, we cannot explain all of these observations, but examination of other enzyme forms (i.e., rbc or serum cholinesterase) may shed some light on the matter.

The relationship of DFP or DNP dose to response of immobilized eel AChE (Figure 20) has possible applications as an in vitro standard screening technique for new inhibitors or as an enzymatic assay for purity or stability studies of organophosphates.

Extending our assay to incorporate brain slices as a functional screening technique showed that membrane bound enzyme (Figure 21) behaved in the same manner as immobilized enzyme as an approximate model for in vivo systems. Two potential problems with this technique are the instability of brain tissue with time and variability in the amount of surface AChE exposed. In addition, the limited amount of AChE exposed forces the experimenter to work with low levels of activity and magnifies activity measurement problems.

b. In Vivo

The low ED_{50} of 5-iodo-2-PAM (5a) in mice challenged with DFP was unexpected in light of the in vitro results, which showed it to be less active than 2-PAM. A possible explanation for this observation could be the slightly better partition coefficient of 5-I-2-PAM, which would favor better CNS penetration. However, 5-I-2-PAM could not be detected in the brain at the levels administered, while 2-PAM was detectable.

No systematic LD_{50} data were collected for the substituted 2-PAM's with the exception of 2-PAM and pro-2-PAM. The LD_{50} of pro-2-PAM was approximately 210 mg/kg and that of 2-PAM was 125 mg/Kg.

An observation concerning the time of regenerator administration was made. It was found that animals injected with 2-PAM at times up to 3 minutes post-DFP-challenge had better survival rates than those injected immediately after DFP administration.

Monitoring of mouse CNS AChE levels following DFP administration (Figures 22-24) indicated a great deal of variability. Although more detailed time course studies of the loss of AChE activity following intoxication could provide some useful information, these studies are not work pursuing because of the technical difficulties.

The development of an effective, universally applicable HPLC assay for pyridinium oximes is a significant breakthrough; this is the first time brain levels of 2-PAM have been detected by nonradiometric techniques. The non-silica based reversed-phase packing material allowed flexibility and tailoring in elution pH profiles. The strongest UV absorption of the pyridinium oxime could be selected - acidic or basic conditions - with no fear of column packing degradation due to extreme pH. The alternative required synthesis of radiolabeled parent quaternary oximes and their prodrug forms. There are no radioactive waste disposal

or accountability problems, and considerable time and manpower savings are associated with the use of this HPLC assay. Radiotracer techniques can afford greater sensitivity at low dose administrations of pyridinium oximes; however, at 100 mg/kg injection levels of 2-PAM, measurable amounts could be detected in the brain even when a 1% residual blood volume containing 2-PAM was taken into account.

F. Recommendations

Our priorities are as follows:

1. Chemistry

a). Perform large scale syntheses (2-4 g) of 3/5-bromo-, 3/5-chloro- and 3/5-carboxamido-2-PAM's 5b, 6b, 5c, 6c, 5e and 6e (Figures 5-8). Collect reproducible analytical and spectroscopic data for all new compounds. If in vitro screening for AChE regenerating activity indicates sufficient efficacy, whole animal survival studies will be performed. Should the parent quaternary oximes prove sufficiently active, the dihydro- or prodrug form will be synthesized and evaluated in vivo.

b). Continue efforts toward the synthesis of stable dihydro-prodrug forms of the 3/5-iodi-2-PAM's and the bromo-, chloro- and carboxamido-substituted pyridinium oximes as indicated by biological screening.

c). Continue investigations in the double latentiation approach (Figure 9). Analogs of 3 where -X is "ideal" (i.e., where X is relatively nontoxic, labile enough to eliminate at physiologic pH's and nucleophilic enough to form the desired tetrahydro-addition product) will be synthesized. We will examine closely the conversion rates to 2-PAM in vitro in a pH window of 6-8 monitoring not only the appearance of 2-PAM but any nonquaternary decomposition products. The most favorable candidates will then be tested in vivo.

d). Continue determination of pKa and partition coefficient (octanol/water and octanol/buffer) data for both parent quaternary oximes and prodrug forms.

2. Biology

a). Extend investgiations into immobilized cholinesterase assays by including human serum and rbc, bovine rbc and horse serum cholinesterases. Compare the inhibition and reactivation properties of these various cholinesterases and identify the most appropriate form for in vitro screening assay protocol.

b). Continue investigations into the use of brain slices (human and animal) and the Ellman method[28] as a screening technique for measuring the effectiveness of reactivators on intact tissue containing organophosphate- inhibited AChE.

c). Continue animal survival studies of the most promising parent quaternary oximes and their prodrug forms - lethal organophosphate challenge/ED_{50} determinations.

d). Continue tissue distribution studies for the most active parent quaternary pyridinium oximes and their prodrug forms, using the HPLC technique developed in our laboratory.

e). Initiate the use of soman (GD) in biological screening. We are now scheduled to receive a shipment of dilute agent.

G. Experimental Methods

1. Equipment and Reagents

Electron impact mass spectra were recorded on either a Varian-MAT CH-5 or Riber R-10-10 mass spectrometer with RDS data system for computer analysis and spectra printout. NMR were obtained with either a Varian T-60, Hitachi Perkin-Elmer R-24B or Varian FT-80a and were run in 1% TMS/$CDCl_3$ unless otherwise noted. The IR were obtained on either a Beckman IR-33 or AccuLab-4 spectrometer, and samples were run as either neat films or KBr pellets (1:100). UV-Vis spectra were recorded on either a Cary 219 or Beckman DU-5 spectrophotometer. HPLC determinations were performed on a Beckman 342 system (112 pumps, 420 controller and 340 organizer), a Kratos 769Z variable UV detector and a Spectra-Physics 4270 recording integrator. Separations were performed on the following reversed-phase columns: a 5 mm x 15 cm Ultrasphere 5 μm ODS RP, 5 mm x 10 cm BrownLee Spheri-5 5 μm RP, and 5 mm x 20 cm BrownLee PRP-1 10 μm RP, all with matching 3 cm guard columns. Normal phase analysis was performed on an Alltech 0.5 x 25 cm silica gel 10 μ column with 3 cm guard column and preparative scale purifications on an Alltech 1 x 25 cm RSIL 10 μ column with a 5 cm guard column. Ionization constants and pH adjustments were performed potentiometrically, using an Orion Research model 399A pH meter equipped with a gel filled combination electrode. Melting points were obtained as either capillary melting points (uncorrected) on a Thomas-Hoover apparatus or as micromelting points (corrected) on a Fisher-Johns melting point stage. The AChE assay was equipped with a Gilson Minipuls 2 variable speed peristaltic pump and a Gilson HM Holochrome variable UV-Vis flow detector.

The chemicals and solvents were generally reagent or HPLC (Chromatography) grade unless purity was not crucial. Thin layer chromatography was carried out on Analtech SG GHLF 250 μm, Analtech Woelm SGF 1000 μm or EM SG-60 F254 200 μm plates. Liquid chromatography sorbents were either Brinkman EM SG-60 70-230 mesh or Woelm silica 63-200 mesh. Acetylcholinesterase (electric eel organ) was obtained commercially purified from either Worthington (1000-1400 units/mg) or Boehringer Mannheim (1000 units/mg). Acetylthiocholine, dithiobis(nitrobenzoic acid) (DTNB), 3-[N-morpholino]propanesulfonic acid (MOPS), diisopropylfluorophosphate (DFP), diethyl-p-nitrophenylphosphate (DNP) and TMB-4 (8) were obtained from Sigma and were used without further purification. DFP degrades slowly, even when stored at 4°C, and was replenished with a fresh supply at regular intervals to insure consistency. The low density polyethylene beads (4mm, precision) were supplied by Precision Plastic Ball Co., Chicago, IL. MINA was purchased from Pfaltz and Bauer, but required purification to homogeneity by LC (SG Woelm, 8% acetone/methylene chloride v/v). Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and are within 0.4% of theoretical values unless otherwise noted.

a. Ionization Constant Determinations

The general procedure required the accurate preparation of .005-.01 M solution of pyridinium oximes. These oxime solutions were then acidified to pH 3 with 0.005 M hydrochloric acid and titrated with 0.005 M

sodium hydroxide. The pH was monitored potentiometrically with a pH meter and combination electrode. Data plots of pH versus volume of sodium hydroxide added were constructed and pKa's were read from titration inflection points.

Partition Coefficient Determinations

The partition coefficients were determined by taking a 1.5 ml solution of quaternary oxime (10^{-5} - 10^{-3} M) in either deionized water or 0.1 M MOPS (pH 7.4) and vortexing 0.5 minute with an equal volume of octanol (spectrograde, presaturated with water). The phases were separated and clarified by centrifugation and the concentrations in octanol determined spectrophotometrically.

2. Chemistry.

3-Iodo-2-picoline (16) and 5-Iodo-2-picoline (17)

A modification of the method of Riley and Perham was employed.[38] A 265 g (1.04 mol) portion of freshly ground iodine was dissolved in 485 ml of 30% oleum over one hour. The dropwise addition of 100g (1.07 mol) of 2-picoline to the reaction mixture via addition funnel proceeded over one hour. The reaction was brought to 150°C for 1.5 hours during which time a vigorous evolution of gas occurred. The reaction was cooled to 120°C and maintained for 12 hours. The mixture was reheated to 150°C for 3 additional hours, then cooled to room temperature. The reaction was poured over 1800 ml of ice and neutralized with sodium carbonate. The neutral aqueous mixture was extracted with 2 x 500 ml of ethyl acetate. The organic extracts were washed twice with brine, dried over sodium sulfate, filtered and flashed to a black oil. A vacuum distillation, b.p. 82-100°C at 5mm (rpt bp 108-109, 18 mm, 17, ref. 38), gave 62.3 g (27%) of 16 and 17 as a yellowish liquid. The roughly equimolar mixture could be partially resolved by LC (SG 70-230 mesh, 10% ethyl acetate/ methylene chloride v/v, load ratio 1:100) and TLC (SG, ethyl ether) Rf 0.57 (17) and 0.67 (16).
17 yellow oil; NMR: δ 2.70 (s, 3H, CH_3), 6.75 (dd, 1H, Ar5H), 7.88 (dd, 1H, Ar4H), 8.38 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 66 (M^+-CN,I), 51 (M^+-CH_3, CN,I).
16 yellow-green crystals, mp (corr) 46-48°C; NMR: δ2.44 (s, 3H, CH_3), 6.88 (d, 1H, Ar3H), 7.78 (dd, 1H, Ar4H), 8.62 (d, 1H, Ar6H), $J_{4,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 65 (M^+-HCN,I), 50 (M^+-CH_3, HCN,I).

3-Iodo-2-picolinealdehyde (18) and 5-Iodo-2-picolinealdehyde (19)

Employing a modification of the method of Markovac et al.[11], 11.9 g (54 mmol) of purified 16 and 17 was mixed with 13.3 g (52 mmol) of iodine. A solid picoline-iodine complex formed, which was gently melted, stirred to homogeneity and allowed to resolidify. The cake was broken up and dissolved in 25 ml of dimethylsulfoxide. The solution was added dropwise via an addition funnel to 30 ml of dimethylsulfoxide, which had been preheated to 150°C. The reaction mixture was maintained at 150-160°C for 40 minutes,during which time evolving dimethylsulfide was trapped in a 20% perchloric acid bubbler. The reaction was cooled to room temperature,

neutralized with a saturated sodium bicarbonate solution and extracted exhaustively with ethyl ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and flashed to 13 g of a viscous black tar. The viscous tar was extracted with several portions of hexane. The hexane extracts were concentrated and loaded onto a silica gel column (70-230 mesh, 1:100 load ratio) and eluted with 10% ethylacetate/methylene chloride (10-12 ml/min). The overall yield was 8.7 g (69%), of which 3.2 g (25%) was pure 5-iodo isomer 19, 3.5 g (28%) pure 3-iodoaldehyde 18 and the remainder a mixture of isomers; tlc (SG, 10% ethyl acetate/methylene chloride v/v) Rf 0.48 (18) and 0.65 (19). Yields ranged from 30-69%. The individual isomers could be further purified by recrystallization from hexane.

18 - needles, pleasant minty odor, mp (corr) 67-69°C; IR: (KBr) 2860 cm^{-1} (CHO), 1700 (CO); NMR: δ 7.15 (dd, 1H, Ar5H), 8.32 (dd, 1H, Ar4H), 8.73 (dd, 1H, Ar6H), 9.83 (s, 1H, CHO), $J_{4,5}$=8 Hz, $J_{4,6}$=2 Hz, $J_{5,6}$=5 Hz (1% TMS/CCl_4); MS: m/e 233 (M^+), 205 (M^+-CO), 78 (M^+-CO, I); Anal. C,H,N.

19 - mp (corr) 112-113°C; IR: (KBr) 2840 cm^{-1} (CHO), 1695 (CO); NMR: δ 7.67 (d, 1H, Ar3H), 8.18 (dd, 1H, Ar4H), 8.98 (d, 1H, Ar6H), 9.96 (s, 1H, CHO), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (1% TMS/CCl_4); MS, m/e 233 (M^+), 205 (M^+-CO), 204 (M^+-CHO), 149 (M^+-CO,I, HCN); Anal. C,H,N.

3-Iodo-2-pyridine carbaldoxime (20)

A saturated aqueous solution of 0.015 g (0.21 mmol) hydroxylamine hydrochloride was neutralized with sodium carbonate and added to a methanolic solution of 0.021 g (0.09 mmol) of aldehyde 18. Within a short time a fine precipitate was evident. The solution was warmed gently to boiling and allowed to cool with stirring overnight. The white needles were collected by suction filtration, the supernate concentrated and a second crop of product collected. The combined crops afforded 0.019 g (82%) of oxime 20, which was recrystallized from ethanol. Yields ranged from 42-82%.

20 - mp 235.5-238.5 (corr); IR: (KBr) 3400 cm^{-1} (brd, OH), 1610 (CH=N); NMR: δ 7.15 (dd, 1H, Ar5H), 8.34 (dd, 1H, Ar4H), 8.61 (m, 2H, Ar6H and CHNOH), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 205 (M^+-CHNO), 204 (M^+-CHNOH), 104 (M^+-I, OH), 91 (M^+-NO, I); Anal. C,H,N.

5-Iodo-2-pyridine carbaldoxime (21)

Preparation of 21 was as described for 20. A 0.73 g (3.1 mmol) portion of 19 was reacted with 0.65 g (9.3 mmol) of hydroxylamine hydrochloride to give 0.69 g (89%) of 21, which could be recrystallized from absolute ethanol.

21 - colorless needles mp (corr) 211-214°C; IR: (KBr) 3400 cm^{-1} (brd, OH), 1560 (CH=N); NMR: δ 7.64 (d, 1H, Ar3H), 8.00 (dd, 1H, Ar4H), 8.12 (s, 1H, CHNOH), 8.79 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 204 (M^+-CHNOH), 121 (M^+-I), 104 (M^+-I,OH), 94 (M^+-I,HCN), 91 (M^+-I,NO); Anal. C,H,N.

3-Iodo-2-pyridine carbaldoxime methiodide (6a)

A modification of the procedure of Poziomek, Hackley and Steinberg was used.[33] A 1.60 g (6.45 mmol) portion of oxime 20 was dissolved in

approximately 100 ml of methylethylketone in a 250 ml pressure bottle. Immediately after purging the head space with nitrogen, 4.0 ml (0.064 mol) methyliodide was added and the vessel sealed with a teflon plug. The vessel was heated in an oil bath at 50°C for 24 hours and cooled to room temperature, and the product was suction-filtered and washed. The yellow-orange needles, 1.92 g (76%), required no further purification.
6a - yellow-orange needles, mp (corr) 195-205°C (dec); IR: (KBr) 3200 cm^{-1} (brd OH), 1620 (CH=N); NMR: δ 4.31 (s, 3H, CH_3), 7.68 (dd, 1H, Ar5H), 8.41 (s, 1H, CHNOH), 8.83 (d, 1H, Ar6H), 8.96 (d, 1H, Ar4H), $J_{4,5}$=8Hz, $J_{5,6}$=6Hz (D_2O/D_6-DMSO with H_2O reference).

5-Iodo-2-pyridine carbaldoxime methiodide (5a)

Preparation of 5a was as described for 6a. Starting with 4.61 g (0.0:86mol) of 21 and 34 ml (0.55 mol) of methyl iodide, the reaction was run in 150 ml absolute ethanol for 3 days at 75°C. A bright yellow crystalline solid 5a, 5.70 g (78%) was isolated. A second crop was obtained by concentrating filtrate and adding ethylacetate.
5a - mp (corr) 220-223°C (dec); IR: (KBr) 400 cm^{-1} (brd OH), 3:00-2730 (oxime), 1620 (C=N); NMR: δ 4.37 (s, 3H, CH_3), 8.18 (d, 1H, Ar3H), 8.67 (s, 1H, CHNO), 8.89 (dd, 1H, Ar4H), 9.20 (d, 1H, Ar6H), $J_{3,4}$=9H, $J_{4,6}$=2Hz (D_2O/D_6-DMSO, H_2O reference); Anal. C,H,N.

3-Iodo-2-pyridine carbaldoxime methochloride (6a)

The halide exchange employed was the method of Kondritzer et al.[40] Dowex-1 resin (chloride form) was rinsed with methanol and repeatedly with deionized water. The final water rinse was tested with 1% silver nitrate solution to ensure that it was free of halogens. A 4.7 g (12 mmol) portion of 6a as the iodide salt was stirred for 4 hours with 150 g of resin in a minimum amount of water. The suspension was filtered and the filtrate flashed to a residue. The resulting chloride salt was recrystallized from ethanol/ethylacetate to afford 2.5 g (69%) of colorless crystals.
6a (chloride form) mp 198-202°C (dec); Anal. C,H,N.

5-Iodo-2-pyridine carbaldoxime methochloride (5a)

Preparation of 5a (chloride form) was as described for 6a above. Starting with a 5.5 g (14 mmol) portion of 5a (iodide form), a glassy residue of the desired product was obtained. The salt could be recrystallized from either ethanol/ethylacetate to afford 3.05 g (72%) of material or water to give 2.70 g (64%) of 5a.
5a (chloride form) mp (corr) 193-195°C (dec); Anal. $C_7H_8N_2OClI \cdot 1/2$ CH_3CH_2OH calc. C,29.88 H,3.44 N,8.71; fnd C,29.44 H,3.52 N,8.57; mp (corr) 198-200°C (dec); Anal $C_7H_8N_2OClI \cdot 1/2\ H_2O$ C,H,N.

3-Bromo-2-picoline (22) and 5-Bromo-2-picoline (23)

A modification of the technique of Riley and Perham[38] was employed. A 35 ml (0.34 mole) portion of 2-picoline was added slowly to 125 ml of 30% oleum. After cooling, 25 ml (0.48 mole) of bromine was added and the reaction heated in a capped pressure bottle at 100°C for 4 days. The cooled reaction mixture was poured over 1000 ml of crushed ice

and was neutralized with solid sodium carbonate. The neutralized aqueous layer was extracted with 2 x 300 ml portions of ethylacetate. The ethylacetate layer was washed with water, dried over sodium sulfate, filtered and evaporated to a residue. The residue was distilled under reduced pressure (3 mm) and resulted in a 23.04 g (38%) portion of colorless oil (b.p. 50-57°X) containing both 22 and 23. Analytical samples of each isomer were prepared by column chromatography (silica gel, 70-230 mesh, load ratio 1:200, 10% ethylacetate/methylene chloride (v/v). Tlc (SG, 10% ethylacetate/methylene chloride v/v) R_f 0.24 (22) and 0.30 (23).

22 - oil; IR (film) 3040 cm^{-1} (Ar-H), 2980 (CH_2-H); NMR: δ 2.58 (s, 3H, CH_3), 6.95 (dd, 1H, Ar5H), 7.73 (dd, 1H, Ar4H), 8.32 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz (TMS/CCl_4).

23 - mp. (uncorr) 29-31°C; IR (melt) 3040 cm^{-1} (Ar-H), 2910 (CH_2-H); NMR: δ 2.42 (s, 3H, CH_3); 6.92 (d, 1H, Ar3H), 7.57 (dd, 1H, Ar4H), 8.43 (d, 1H, Ar6H) $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4).

3-Bromo-2-picolinealdehyde (24) and 5-Bromo-2-picolinealdehyde (25)

Employing a modification of the method of Markovac et al.[11], 10.1 g (.058 mole) of 22 and 23 was mixed with 14.8 g (0.059 mole) of freshly ground iodine. The resulting solidified complex was dissolved in 58 ml of DMSO and treated as described in the synthesis of 18 and 19. The resulting crude product mixture was purified by column chromatography (silica gel, 70-230 mesh, 10% ethylacetate/methylene chloride) to afford 1.1 g (10%) 24, 2.20 g (20%) 25 and 3.15 g (29%) of the mixture.

24 - yellow needles, mp. (uncorr) 64-65°C; IR: 3040 cm^{-1} (Ar-H), 2810 (CHO), 1720 (CO); NMR: δ 7.50 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), 10.03 (s, 1H, CHO), $J_{4,5}$=9Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/CCl_4/d_6-Acetone); MS: m/e 187, 185 (M^+), 159/157 (M^+-CO), 78 (M^+-Br,CO); Anal. C,H,N.

25 - yellow needles, mp. (corr) 90-93°C; IR: 3050 (Ar-H), 2850 (CHO), 1700 (CO); NMR: δ 7.75 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.78 (d, 1H, Ar6H), 9.92 (s, 1H, CHO), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4); MS: m/e 187/185 (M^+), 159/157 (M^+-CO), 78 (M^+-Br,CO); Anal. C,H,N.

3-Bromo-2-pyridine carbaldoxime (26)

Preparation of 26 was as described for 20. A 0.38 g (2.0 mmole) portion of 24 was reacted with 0.20 g (2.9 mmole) of hydroxylamine hydrochloride to afford 0.37 g (90%) of 26.

26 - tan needles, mp. (corr) 231-234°C; IR: 3400 (OH), 3130-2760 (oxime); NMR: δ 7.32 (dd, 1H, Ar5H), 8.10 (dd, 1H, Ar4H), 8.42 (s, 1H, CHON), 8.61 (dd, 1H, Ar6H), 11.88 (s, 1H, NOH), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/d_6-DMSO/d_5-acetone); MS: m/e 202, 200 (M^+), 158, 156 (M^+-CHNOH).

5-Bromo-2-pyridine carbaldoxime (27)

Preparation of 27 was as described for 20. A 0.41 g (2.2 mmole) portion of 25 was reacted with 0.25 g (3.6 mmole) of hydroxylamine hydrochloride to give 0.42 g (95%) of desired oxime 27.

27 - tan needles, mp. (corr) 209-211°C; IR: 3400 cm^{-1} (OH), 3100-2790 (oxime); NMR: δ 7.73 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.03 (s, 1H, CHNO), 8.63 (d, 1H, Ar6H), 11.45 (s, 1H, NOH), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz

(TMS/d_6DMSO/$d_6$acetone); MS: m/e 202/200 (M^+), 158, 156 (M^+-CHNOH); Anal. C,H,N.

5-Bromo-2-pyridine carbaldoxime methiodide (5b)

Preparation of 5b was as described for 6a. Starting with 0.46 g (2.3 mmole) of 27 and 3.5 ml (5.6 mmole) of methyl iodide, the reaction was run in 20 ml of methylethylketone for 24 hours at 90°C. A bright yellow crystalline solid, 5b, 0.35 g (45%), was isolated. The use of ethylacetate as a reaction solvent results in yields up to 72%.
5b - yellow needles, mp. (uncorr) 213-215°C; IR: (KBr) 3400 cm^{-1} (OH), 3100-2710 (oxime); NMR: δ 4.53 (s, 3H, CH_3), 8.39 (d, 1H, Ar3H), 8.68 (s, 1H, CHNO), 8.82 (dd, 1H, Ar4H), 9.58 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/d_6-DMSO/ d_6-acetone).

5-Bromo-2-pyridine carbaldoxime methochloride (5b)

Preparation of 5b (chloride form) was as described for 6a (methochloride salt). Starting with a 0.35 g (1 mmol) portion of the methiodide salt, 0.18 g (70%) of the desired methochloride salt was isolated as colorless needles.
5b - (chloride form) mp. 199-201°C; IR: (KBr) 3400 cm^{-1} (OH), 3100-2710 (oxime); NMR: δ 4.40 (s, 3H, CH_3), 8.33 (d, 1H, Ar3H), 8.68 (s, 1H, CHNO), 8.72 (dd, 1H, Ar4H), 9.12 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TSP/D_2O).

3-Iodo-2-pyridinealdehyde diethylacetal (28)

Employing a modification of Shek et al.[21] 1.22 g (5.2 mmol) of 3-I-picolinealdehyde (18) and 2.5 ml (15.0 mmol) triethylorthoformate were brought to a reflux under a nitrogen atmosphere in 10 ml of absolute ethanol saturated with HCl gas. The reaction was cooled to room temperature after 3-4 hours and nitrogen bubbled through the solution for 10-15 minutes to vent off excess HCl. The solution was transferred to a round bottom flask and flashed to the ammonium salt. A 25 ml portion of saturated $NaHCO_3$ solution and 50 ml of ether were added to the residue and stirred. The ether layer was removed and washed with several small portions of water and with 2x15 ml of brine, dried over Na_2SO_4, filtered and flashed to a dark oil. The oil was loaded onto a short column of silica gel (70-230 mesh) and eluted with dry acetone. The resulting light tan oil was dried at 35-55 mm Hg for several hours - 1.48 g (92% theoretical) of desired acetal 28.
28 - tan oil; NMR: δ 1.25 (t, 6H, CH_2CH_3) 3.7 (q, 4H, OCH_2CH_3), 5.75 (s, 1H, $CHOCH_2$), 6.95 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.6 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/CCl_4); MS: CI-NH_3, 308 (M^++1), 263 (M^++1 - OCH_2CH_3).

5-Iodo-2-pyridinealdehyde diethylacetal (29)

Preparation of 29 was as described for 28. Starting with a 2.0 g (8.6 mmol) of 19 and 3.5 ml (21 mmol) of triethylorthoformate in 20 ml absolute ethanol, 2.43 g (92%) of 29 was isolated.
29 - tan oil; NMR: δ 1.2 (t, 6H, CH_2CH_3), 3.60 (dq, 4H, OCH_2CH_3), 5.25 (s, 1H, $CHOCH_2$), 7.3 (d, 1H, Ar3H), 8.0 (dd, 1H, Ar4H), 8.7 (d, 1H, Ar6H), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (TMS/CCl_4); MS: CI-NH_3, 308 (M^++1), 263 (M^++1-OCH_2CH_3).

3-Cyano-2-pyridinealdehyde diethylacetal (30)

Using the modified method of Friedman and Shecter,[41] 1.4 g (4.6 mmol) of 28 and 0.41 g (4.6 mmol) of cuprous cyanide were brought to a vigorous reflux under a nitrogen atmosphere in 10 ml dry DMF. The reaction went from colorless to deep red/brown over 6 hours. After cooling to room temperature, 80 ml of 20% aqueous sodium cyanide was added slowly with stirring to the reaction mix. The addition was exothermic as the copper complexes decomposed. The cooled mixture was extracted with several 20 ml portions of ether. Combined ether extracts were washed with 2 x 15 ml water, 2 x 30 ml brine, dried over sodium sulfate, filtered and flashed to a deep red/brown color. The oil was loaded onto a short silica gel column (25 g, 70-230 mesh) and eluted with 5% ethylacetate/methylene chloride v/v to decolorize. The resulting light tan oil, 0.42 g (44%), was single spot to TLC (SG, 5% ethylacetate/methylene chloride).
30 - tan oil; IR: 2205 cm^{-1} (CN); NMR; δ 1.25 (t, 6H, $CH_2\underline{CH}_3$), 3.70 (dq, 4H, $OCH_2\underline{CH}_3$), 5.65 (s, 1H, $\underline{CHOCH}_2$), 7.35 (dd, 1H, Ar5H), 8.0 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz, $J_{4,5}$=5Hz (TMS/$CDCl_3$); MS: CI-NH_3, m/e 207 (M^++1), 161 (M^++1 - $HOCH_2CH_3$).

5-Cyano-2-pyridinealdehyde diethylacetal (31)

Preparation of 31 was as described for 30. Starting with 2.0 g (6.5 mmol) of 29 and 0.586 g (6.5 mmol) of CuCN in 10 ml dry DMF, 0.96 g (72%) of desired product 31 was isolated as a light tan oil following column clean-up.
31 - tan oil; IR: (film) 2205 cm^{-1} (CN); NMR: δ 1.25 (t, 6H, $CH_2\underline{CH}_3$), 3.70 (q, 4H, $OCH_2\underline{CH}_3$), 5.4 (s, 1H, $\underline{CHOCH}_2$), 7.70 (d, 1H, Ar3H), 8.0 (dd, 1H, Ar4H), 8.8 (d, 1H, Ar6H), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (TMS/$CDCl_3$); MS: CI-NH_3, m/e 207 (M^++1), 162 (M^++1)-OCH_2CH_3).

3-Cyano-2-pyridinealdehyde (32)

A 0.075 g (0.39 mmol) portion of cyano acetal 30 was dissolved in 2 ml absolute methanol along with 0.2 ml 2N HCl and brought to a vigorous reflux for 30 minutes under a nitrogen atmosphere. Volume of the cooled reaction mixture was reduced under vacuum, basified with $NaHCO_3$ and extracted with several small portions of ether. The ether extracts were dried over Na_2SO_4, filtered and flashed to 0.040 g of a light yellow aromatic oil. Tlc indicated two products; therefore, the material was separated into its components via HPLC (0.5 x 25 cm, SG, 10 μ, 25% ethyl acetate/chloroform, 1 ml/min). A very volatile material was collected at R_t of 5 minutes and 0.026 g (50%) of the desired material 32 eluted off the column at 10.6 minutes as a colorless oil.
32 - viscous colorless oil; IR: (film) 2850 cm^{-1} (CHO), 2200 (CN), 1725 (CO); NMR: δ 7.35 (dd, 1H, Ar5H), 8.0 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), 10.4 (s, 1H, CHO), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/$CDCl_3$).

5-Cyano-2-pyridinealdehyde (33)

Preparation of 33 was as described for 32. Starting with 0.10 g (0.48 mmol) of 31, a 0.051 g (80%) portion of the 5-cyano aldehyde 33 was isolated.

33 - IR: (KBr) 2860 cm^{-1} (CHO), 2225 (CN), 1710 (CO); NMR: δ 8.04 (m, 2H, Ar3, 4H), 9.01 (d, 1H, Ar6H), 10.07 (s, 1H, CHO), $J_{4,6}$=2Hz (TMS/CCl_4); MS: m/e 132 (M^+), 104 (M^+-CO), 103 (M^+-CHO), 77 (M^+-CO, HCN).

5-Cyano-2-pyridine carbaldoxime 35)

Preparation of 35 was as outlined for 3-I-2-pyridine carbaldoxime 20. A 0.157 g (1.19 mmol) portion of 33 was reacted with 0.082 g (1.19 mmol) of hydroxylamine hydrochloride to give 0.097 g (55%) of 35 as colorless needles. 35 - mp (uncorr) 190-200°C; IR: (KBr) 2220 cm^{-1} (CN), 1600 (C=N); NMR: δ8.10 (m, 3H, Ar3, 4H and CHNOH), 8.93 (d, 1H, Ar6H)11.81 (s, 1H, CHNOH), (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 147 (M^+), 129 (M^+-H_2O), 117 (M^+-NO), 104 (M^+-CHNO), 90 (M^+-NO, HCN), 76 (M^+-HCN, CHNOH).

3-Carboxamido-2-pyridinealdehyde diethylacetal (37)

The basic procedure of Galat[42] was used. A 0.05 g (0.24 mmol) portion of cyano acetal 30 was dissolved in 2 ml 95% ethanol. A 1 ml volume of wet (water) Dowex-1 resin, prepared as the hydroxide form, was added and the mixture refluxed for 36 hours under a nitrogen atmosphere. The cooled reaction was filtered to remove the resin. The filtrate was flashed to a residue and dried under vacuum to remove water. The resulting colorless oil solidified on standing to afford 0.060 g (quant) of single spot material (tlc, SG, 8% methanol/methylene chloride v/v, Rf .36). 37 - IR: (KBr) 1750 cm^{-1} ($CONH_2$); NMR: δ 1.2 (t, 6H, CH_2CH_3), 3.7 (dq, 4H, OCH_2CH_3), 5.55 (s, 1H, $CHOCH_2$), 7.3 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.6 (dd, 1H, AR6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/d_6-acetone); MS: CI-NH_3, m/e 225 (M^++1), 179 (M^++1-$HOCH_2CH_3$).

5-Carboxamido-2-pyridinealdehyde diethylacetal (38)

Preparation of 38 was as outlined for 37. Starting with 0.25 g (1.21 mmol) of cyano acetal 31 and 1.5 ml wet volume of resin, 0.22 g (81%) of the desired amide 38 was isolated.
38 - tan solid, mp (uncorr) 112-115°C; IR: (KBr) 1750 cm^{-1} ($CONH_2$); NMR: δ 1.25 (t, 6H, CH_2CH_3), 3.6 (q, 4H, CH_2CH_3), 5.55 (s, 1H, $CHOCH_2$), 7.1 (d, 1H, Ar3H), 8.3 (dd, 1H, Ar4H), 9.1 (d, 1H, Ar6H), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (TMS/d_6-acetone).

5-Carboxamido-2-pyridinealdehyde (40)

Preparation of 40 was as described for 3-cyano aldehyde 32. Starting with 0.076 g (0.34 mmol) of 38, 0.042 g (83%) of 40 was isolated. 40 - IR: (KBr) 1740 cm^{-1} (brd, CO); NMR: δ 7.75 (dd, 1H, Ar3H), 8.0 (dd, 1H, Ar4H), 8.8 (d, 1H, Ar6H), 10.1 (s, 1H, CHO), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (TMS/$CDCl_3$).

5-Carboxamido-2-pyridine carbaldoxime (42)

Preparation of 42 was as outlined for 3-I-2-pyridine carbaldoxime 20. Starting with 0.030 g (0.2 mmol) of 39, 0.035 g (quant) of oxime 42. 42 - tan granular solid, mp (uncorr) 229-231°C (dec), rept. 234-236°C, ref 11.

N-Methyl-2-cyano-1,2,3,6-tetrahydropyridine-2-carbaldoxime (3a)

The procedure of Bodor, Shek and Higuchi[21] was employed startign with 2.5 g (9.5 mmol) of 2-PAM (7); 0.53 g (38%) of 3a was isolated as a light tan solid. The material darkened considerably with time when stored at room temperature in ir. Even when stored under nitrogen, the compound was not especially stable.
3a - mp 101-104°C (rpt 112-114, ref. 21); IR: (KBr) 3200 cm^{-1}(brd OH), 2300 (CN), 1650 (C=N); NMR: δ 2.38 (s, 3H, CH_3), 3.15 (m, 4H, CH_2CH), 5.75 (m, 2H, $CH_2CHCHCH_2$), 7.35 (s, 1H, CHNOH), (TMS/CD_3COCD_3).

N-Methyl-1,2,3,6-tetrahydropyridine-2-carbaldoxime-2-thiocyanate (3b)

A modification of the method of Bodor, Shek and Higuchi[21] was used. Into 10 ml of water was dissolved 1.5 g (15.4 mmol) of potassium thiocyanate and 1.0 g (3.8 mmol) of 2-PAM (7). The solution was degassed with nitrogen and chilled to 0°C and the pH adjusted to 1-2 with concentrated hydrochloric acid. The aqueous solution was layered with 50 ml of ethyl ether and 0.2 g (5.3 mmol) of sodium borohydride was added in one portion. The temperature was maintained at 0°C until the ebullition ceased and then the reaction was allowed to warm to room temperature slowly. The nitrogen atmosphere was maintained and the pH monitored throughout the reaction. The pH leveled off at 7-8 and remained at that figure through most of the reaction. After 4 hours the aqueous layer was saturated with sodium chloride, the ether layer removed and the aqueous layer extracted with a second 50 ml portion of ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and evaporated to 0.240 g (32%) of a light pink solid, 3b. The product darkened considerably with time even when stored under a nitrogen atmosphere.
3b - mp (corr) 94-96°C; IR (KBr) 2110 cm^{-1} (SCN), 1665 (HC=N); NMR: δ 2.35 (s, 3H, CH_3), 3.20 (m, 4H, $CH_2CHCHCH_2$), 5.70 (m, 2H, CH_2CHCH), 7.45 (d, 1H, CHNOH), (TMS/CD_3COCD_3).

N-methyl-1,2,3,6-tetrahydropyridine-2-bromo-2-carbaldoxime (3c)

Preparation of 3c was as described for 3b above. Starting with a 0.264 g (1.0 mmol) portion of 2-PAM (iodide salt form), 0.064 g (29%) of the desired bromine addition product was isolated.
3c - colorless solid, mp. 96-99°C; IR (KBr) 1670 (C=N); NMR: δ 2.35 (s, 3H, CH_3), 3.20 (m, 4H, CH_2 $CHCHCH_2$), 5.72 (m, 2H, CH_2 $CHCH$), 7.30 (s, 1H, CHNO) (TMS/d_6-acetone).

% Conversion Determination - 3 (a-c) to 2-PAM (7)

Samples of 3 (10^{-3} M) were prepared in 150 mM Na_3PO_4 buffer (pH range 6.5-8.5, adjusted with H_3PO_4 or Na_3PO_4). Samples were stirred in a vessel open to atmosphere and aliquots were withdrawn, mixed 1:1 with mobile phase and injected (20 µl on column) at 5, 10, 20, 40, 80, 100, 320 minutes and 24 hours. Identification and quantitation were performed via HPLC, using a reversed-phase C18 column (see Experimental Methods - 1. Equipment and Reagents) eluted with 20% acetonitrile/aqueous paired ion reagents (0.01 M heptane sulfonic acid, 0.001 M tetraethylammonium perchlorate, adjusted to pH 3.5 with acetic acid) at 1.5 ml/minute. 2-PAM exhibited a t_R of 2.7-3 minutes.

3. Bioassay

a. Immobilized Acetylcholinesterase Assay

Purified AChE (E.C.3.1.1.7, Electrophorus electricus) was immobilized using a modification of the technique reported by Ngo, Laidler and Yam[36]. The modifications were as follows: precision ground 4 mm spherical low density polyethylene beads were used; all reactions and washers were conducted using an overhead mechanical stirrer with teflon paddle; treatment of the beads with thionyl chloride, ethylenediamine and required rinses was carred out under a nitrogen atmosphere; the beads were treated with 10% glutaraldehyde for three hours with one solution change; the attachment of glutaraldehyde to the bead could be monitored for completeness by incubating the beads in the presence of DTNB, since any noncapped amino groups on the bead surface reacted with DTNB, resulting in the generation of a chromophore which was monitored at 412 nm; the enzyme was loaded onto the beads in a two hour room temperature incubation; and MOPS buffer (0.1 M, pH 7.8) replaced phosphate buffer throughout.

Beads containing immobilized enzyme were stored at -16°C in MOPS buffer containing 40% glycerin. The stability of the enzyme activity was effected by freeze-thaw cycles; therefore, the beads were frozen in small batches. The activity was stable for up to four months when stored as described. Enzyme activity per bead averaged 0.5 unit/bead; however, significant bead to bead variation was observed. Under normal assay conditions (0.1 M MOPS buffer, pH 7.8, 37°C) 1.5-2% loss of enzyme activity was observed.

b. Measurement of AChE Activity

The enzyme activity assay was based on the Ellman technique.[35] Acetylthiocholine (in 90% ethanol) and DTNB (in 95% ethanol) were added to 30 ml of MOPS buffer as above to given concentrations of 1×10^{-3} M and 5×10^{-4} M, respectively. Figure 11 is a schematic of the closed loop flow-through system employed in the assay. The peristaltic pump was routinely run at 5.8 ml/min and the column effluent monitored at 412 nm. The column was a polypropylene cylinder which contained immobilized enzyme beads packed with alternating glass beads which optimized surface area and flow characteristics. A solution in the flask containing substrate and chromogen was directed via switching valves through the column. The flask contents were cycled such that a stable baseline rate of enzyme activity was observed on the recorder trace. An excess of DFP (0.572 mM) was added and after all enzyme activity ceased (approximately 5 minutes), the system was flushed with fresh buffer via switching valves.

Reactivators were routinely prepared in 200 ml volumes; however, some of the highest concentrations of 2-PAM and MINA were prepared in 100 ml volumes to conserve material. As mentioned earlier, phosphorylated oximes are potent enzyme inhibitors. To reduce the possibility of the phosphorylated oximes inhibiting the reactivated enzyme, the eluent was passed out of the system for the first five minutes of exposure to the reactivators. A fresh solution of reactivator was then recycled for the remaining exposure time. We observed no difference between the above procedure and pumping reactivator through the column in a single-pass

fashion for the entire 45 minute exposure time. In the case of 2-PAM, we also examined the concentrations of reactivator (295 nm), via HPLC, both entering and exiting the column and found no difference.

The detector output was recorded as absorbance units vs time which was converted to rate of substrate hydrolysis using the conversion of Ellman.[28]

$$\frac{\Delta \text{ Abs/min}}{1.36 \times 10^{-4}} = \text{mol thiocholine/l.min.}$$

The substrate concentration routinely used in the assay was at saturation level, and the flow rate was an intermediate value. There was no apparent effect of flow rate on the regeneration of DFP-inhibited immobilized enzyme.

The closed loop flow-through assay system illustrated in Figure 11 was also used to monitor AChE activity in brain homogenates, purified soluble eel enzyme and mouse brain slices in place of immobilized eel enzyme. In the case of purified eel enzyme, an aliquot sufficient to generate measurable cholinesterase activity was added to the circulating milieu and could be deactivated with DFP in the usual manner. Whole brain or brain sections of freshly decapitated mice were homogenized with 1 ml water and an aliquot of crude homogenate was assayed as purified eel enzyme was. In addition, the AChE activity of mice injected with doses (2,4,6 mg/kg) of DFP could be monitored in the presence and absence of reactivators (Figures 21-24). Animals were sacrificed at 3 or 5 minutes, dissected and the whole brain or section was homogenized and assayed for AChE activity. In addition, brain slices (50-100 mg) were also substituted for immobilized AChE and proved to be an effective enzyme source for screening purposes. The amount of exposed enzyme in a brain section was small, however, it could be inhibited and regenerated in a manner identical to that used in the immobilized enzyme assay.

Dose response lines for DFP and DNP inhibited immobilized eel AChE were developed by adding small amounts of the organophosphates to a set of beads and recording the depressed rates of hydrolytic activity. Each concentration of organophosphate required a fresh batch of immobilized enzyme containing beads.

b. In Vivo - Animal Survival Studies

Adult male mice (CF1 outbred), 25-35 g, obtained from Sasco, Inc., Omaha, NE, were used. Experiments were performed using same shipment animals for consistency. Freshly prepared DFP (6 mg/kg, 2 x LD_{50}) in water was injected sc (20-35 µl). Reactivators (up to 100 mg/kg) were prepared in water and injected into the left hind limb im (100 µl, 1% body volume) except for pro-2-PAM (4) which was administered iv (tail, 20 second infusion) in freshly prepared and deoxygenated citric acid buffer (50 mM, pH 3.5).[29] Groups of four or five animals/dose and four dose levels were used. Survivals were counted at 24 hours. Calculations for LD_{50}, ED_{50} and 95% confidence limits were according to Weil.[43]

For the experiments outlined in Figures 22-24, mice were injected with varying doses of DFP (sc). At various times post-injection, as well as at death, the animals were sacrificed by decapitation, the brains removed and the appropriate brain section assayed for residual AChE activity using the modified Ellman method.

c. HPLC Assay for Pyridinium Oximes

The reversed-phase PRP-1 column (see Experimental Methods - 1. Equipment and Reagents) was eluted with 0.1 M Na CO (pH 10.5) at 1 ml/min with detection at 335 nm for 2-PAM. Detection could be tailored to individual oximes. All tissues, except blood, were prepared by rotary high speed homogenization in 1 ml water. A 40-50 l portion of concentrated perchloric acid was added as a precipitant and the homogenate was centrifuged for 15-20 minutes at 1500 g. The clear supernate was filtered (0.45 m), basified, and frozen. Thawed samples were recentrifuged and 20 l portions injected on the HPLC. Blood samples were collected and 40 l of 15% EDTA added to prevent coagulation. These samples were centrifuged and the serum removed. A 10 l portion of perchloric acid was added to the serum, the sample was centrifuged and the supernate was filtered and basified. Biological materials eluted first (2.5-3.5 minutes), followed by the oximes. T_R of 2-PAM was 4.5 minutes, depending on peak size, and liver/brain occasionally caused peak spreading. Detection limits were in the 40 pmole range (i.e., 10 ng 2-PAM on column).

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